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Transplantation of cultured corneal endothelial cells: Towards clinical application

Spinozzi D.

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Daniele Spinozzi

TRANSPLANTATION OF CULTURED CORNEAL ENDOTHELIAL CELLS: TOWARDS CLINICAL APPLICATION

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Transplantation of cultured corneal endothelial cells: towards clinical application

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TRANSPLANTATION OF CULTURED CORNEAL ENDOTHELIAL CELLS: TOWARDS CLINICAL APPLICATION

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CHAPTER 1

Introduction and outline of the thesis

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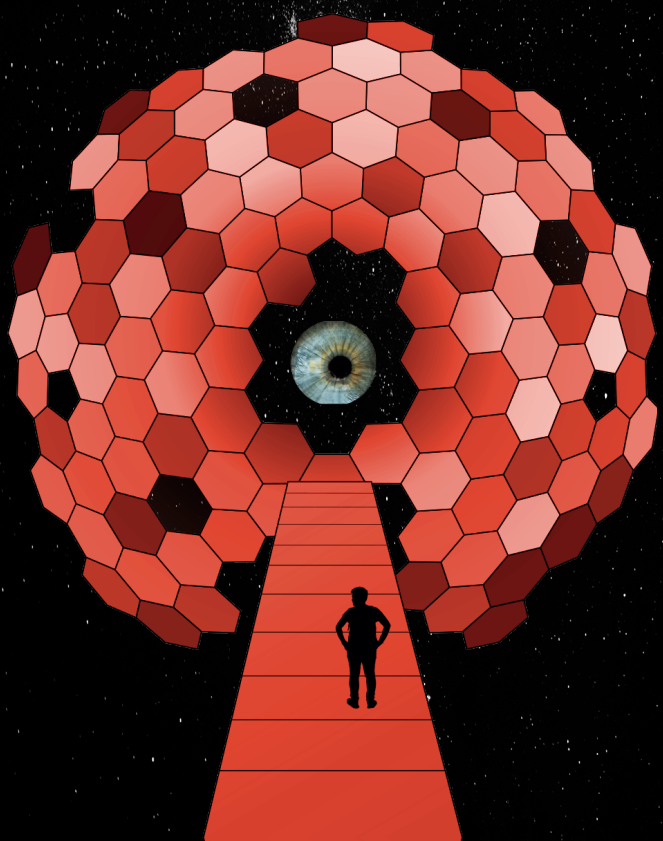

INTRODUCTION

Corneal endothelium: structure, functions and pathologies

The human cornea is an avascular tissue with a crucial function in vision by serving, amongst others, that supports the light stream through the lens and the retina and as a barrier to the external environment. The cornea is organized in three main cellular layers: the epithelium, the stroma and the endothelium, while the limbus acts as a stem cell reservoir for the corneal epithelium.[1] The human corneal endothelium can be histologically identified as resembling simple cuboidal tissue and it marks the posterior part of the cornea.[2] This tissue is formed by a single layer of tightly-packed cells that display an hexagonal morphology on the apical side, facing the aqueous humor, while the surface on the basal side towards the Descemet's membrane, a basement membrane formed by secretions from the endothelium itself, is irregular (**Figure 1**).[3-5]

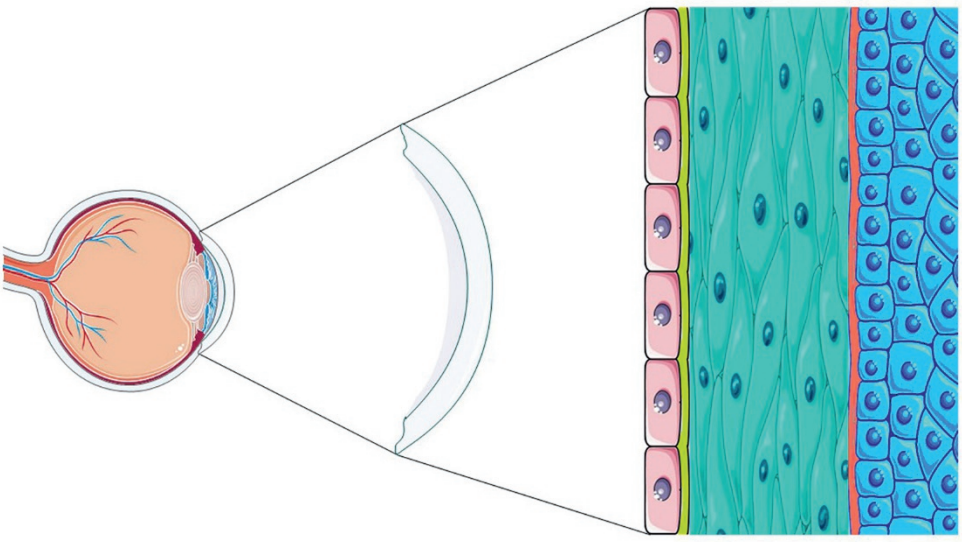

Figure 1: The human cornea. Schematic representation of the human cornea and its position within the human eye. Several layers can be distinguished: corneal endothelium (in pink, average thickness of 5 μm), Descemet's membrane (in yellow, average thickness of 10-12 μm), corneal stroma (in green, average thickness of 500 μm), Bowman layer (in red, average thickness of 8-14 μm) and corneal epithelium (in blue, average thickness of 50 μm). Thickness of the corneal layers is not drawn to scale.

Human corneal endothelial cells (hCEC) have an approximate thickness of 5 μm and a diameter of 20 μm and maintain the stromal hydration (fundamental for the preservation of corneal transparency) through transmembrane ion transporters coupled with Na^+/K^+-ATPase and Mg^{2+}-ATPase on the basolateral side of the cells.[6-10] Within the endothelial layer, hCEC shape and migration are maintained by actin filaments,[11] while cadherin and different catenin isoforms are mainly involved in apical cell junctions.[12] Other type of junctions present in the corneal endothelium are tight junctions, associated with the Zonula Occludens (ZO)-1 complex,[13] and gap junctions, responsible of the electrical communication between cells mediated by the connexin-43 protein.[14] As hCEC are not thought to be capable of replicating *in vivo* because they cannot overcome the G1 phase in the cell cycle,[15-17] their density and number decline naturally with the age, at a rate of about 0.6% per year (10.9 cells/mm^2 per year, according to confocal microscopy measurements).[18,19] At birth, the average hCEC density is 3500 – 4000 cells/mm^2,[20] which declines to 2300 cells/mm^2 by age 85.[18] Moreover, hCEC density is reported to be higher in the peripheral and paracentral areas compared to the center of the endothelium.[21] Damaged and dead hCEC are replaced by a mechanism of cell migration of the neighboring cells, that guarantees a restoration of functionality in the affected area but also a lower cell density.[22] Below the arbitrary threshold of 500 cells/mm^2, the endothelium does not have enough pumping power to guarantee a correct corneal hydration, leading to loss of corneal clarity, impairment of visual acuity and, finally, corneal blindness.[23]

Two of the most common pathologies occurring at the corneal endothelial level are Fuchs endothelial corneal dystrophy (FECD), a condition in which extracellular matrix (ECM) deposits called guttae – together with hCEC death – result in impaired vision,[24] and bullous keratopathy, where small vescicles defined bullae arise after endothelial damage and contribute to the formation of a corneal edema.[25] The treatment of corneal endothelial pathologies by corneal transplantation has largely improved over the past few decades and the steps conducted in the field of corneal endothelial transplantation gave the possibility to many people to restore vision and to resume a normal life.[26]

Surgical treatments of corneal pathologies

For many years, the only effective treatment was a full thickness transplant, or penetrating keratoplasty (PK), which requires the removal of all layers of the damaged cornea and the replacement with a donor cornea. This type of invasive surgery requires the application of sutures, that have to stay in place typically up to 1 year. Moreover, the rehabilitation is very slow, as it may take several years to achieve a full recovery.[27] In the late 1990s, a new surgical technique was introduced, named posterior lamellar keratoplasty or deep lamellar endothelial keratoplasty (DLEK). With the replacement of the corneal endothelium through a limbal incision, this technique was considered a breakthrough because less incisions or sutures were needed, thus solving most of the issues related to the classic PK.[28,29] Despite the promising results in terms of fast recovery of visual acuity and contained cell loss at 6 months after surgery,[30] the procedure was still challenging from a technical point of view, as the manual dissection of both the donor and the host stromal beds were required.[31]

In the early 2000s, a more selective technique called Descemet's stripping endothelial keratoplasty (DSEK) was developed. The major difference between DSEK and its precursor was the selective removal (i.e. "stripping") of the host's damaged Descemet membrane together with the endothelium: this step was called "descemetorhexis[32] and was followed by the replacement of the damaged tissue with a thin layer of stroma which has attached the healthy Descemet's membrane and endothelial layer coming from a donor. Once the donor tissue is implanted into the eye, an air bubble that usually lasts a few days supports the new tissue. Strong advantages of this technique compared to PK are a better postoperative vision, a quicker recovery of vision and lower postoperative complication rates.[32-34]

In 2006, Gerrit Melles introduced an even more selective technique for endothelial keratoplasty. This technique was called Descemet's membrane endothelial keratoplasty (DMEK) and represented a milestone in the history of corneal transplantation. Briefly, this approach permits the selective replacement of the damaged corneal endothelium with the Descemet's membrane and healthy endothelium coming from a donor, with no additional layers such as the stroma.[35] Early standardization of the surgical technique facilitated acceptance among surgeons,[36,37] and nowadays this technique represents a very efficient

treatment for the abovementioned corneal pathologies and its application improves visual outcomes and reduces the risk of graft rejection (**Figure 2**).[38-40]

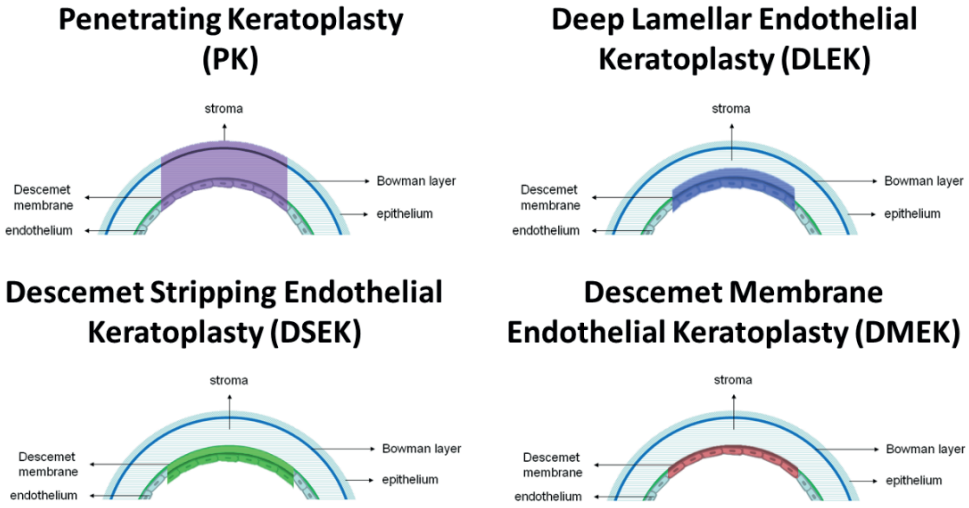

Figure 2: Development of endothelial keratoplasty. For many years, the only possibility to perform a corneal transplantation was penetrating keratoplasty (PK), a technique that required the application of sutures, with consequent very slow recovery of the patient. From the late 1990s, this surgical technique evolved to endothelial keratoplasty with the purpose of selectively removing the damaged endothelium. Scientific and technical progress led to the development of deep lamellar endothelial keratoplasty (DLEK), followed by Descemet stripping endothelial keratoplasty (DSEK) and Descemet membrane endothelial keratoplasty (DMEK), the most selective corneal transplantation surgical technique currently available (Adapted from: Current Treatment Options for Fuchs Endothelial Dystrophy, Editors: Cursiefen, Jun. Chapter 5: Baydoun, Dapena, Melles - Evolution of Endothelial Keratoplasty)

Nevertheless, as for many fields in tissue transplantation, use of endothelial keratoplasty is restricted by a lack of donor tissue available, because of a global shortage of donors. The reasons are multiple: increase in life expectancy, social and religious motivations, strict criteria for tissue release, etc.[23,41,42] Nowadays, it has been estimated that there is only 1 donor cornea available for every 70 patients in need of a transplantation worldwide.[43] As a result, techniques have been developed to use the available donor tissue more efficiently. The latest

surgical approaches explored against the global tissue shortage include Descemet stripping only (DSO), also known as Descemetorhexis without endothelial keratoplasty (DWEK), and hemi- and quarter-DMEK as some of the most promising and innovative ones.[44-46]

DSO has been progressively applied as a treatment for patients affected by central FECD and is based on the removal of the central guttae, without replacement by donor tissue. The rationale behind the implementation of this technique is that, if the guttae are confined to the corneal center, a planned "guttaectomy" to remove the diseased areas would boost the repopulation of this part of denuded stroma by the surrounding healthy hCEC.[47] Varying success rates have been reported for this technique in terms of the restoration of corneal clarity, by re-population of the stripped area by peripheral endothelial cells.[48-50]

Hemi- and quarter-DMEK are corneal transplantation techniques that were introduced by the Netherlands Institute for Innovative Ocular Surgery (NIIOS), with the purpose of increasing the pool of available donor tissue by two and four times, respectively. For the hemi-DMEK, two semicircular endothelial sheets of 12 mm of diameter were obtained from one corneoscleral rim by completely stripping the DM from the posterior stroma and cutting the untrephined graft in half. This way, a hemi-DMEK graft has a comparable surface area to a standard, trephined DMEK graft and two patients could be treated from one donor cornea (**Figure 3A**).[44,51] Results from 10 patients treated with hemi-DMEK surgery showed similar visual outcomes to conventional DMEK surgery up to 4 years of postoperative follow-up. At the same time, the steep decrease in ECD after 6 months could be a consequence of a distinct mechanism of cell migration and ECD measurements taken in different areas compared to the conventional DMEK.[52,53]

The Quarter-DMEK technique went one step further towards the optimization of the donor tissue available, by reducing the size of the DM graft in order to obtain 4 grafts from one donor cornea.[54,55] Briefly, the corneo-scleral button is divided into 4 equal parts and the DM is then completely stripped off, resulting in 4 smaller DM grafts of 5-6 mm along the radial edge (**Figure 3B**). The obtained Quarter-DMEK graft is then transplanted by positioning the graft in the central part of the posterior stroma, inducing the donor endothelial cells to

migrate adjacently to the graft. Best- corrected visual acuity (BCVA) was comparable to a standard DMEK surgery up to 6 months follow-up. The decrease in ECD, despite a steep decline after 6 months, also stabilized thereafter up to 2 years of follow-up.[45,56] Nevertheless, corneas treated with Quarter-DMEK surgery cleared slower compared to a conventional DMEK treatment, especially in the limbal area of the Quarter-DMEK graft.[45]

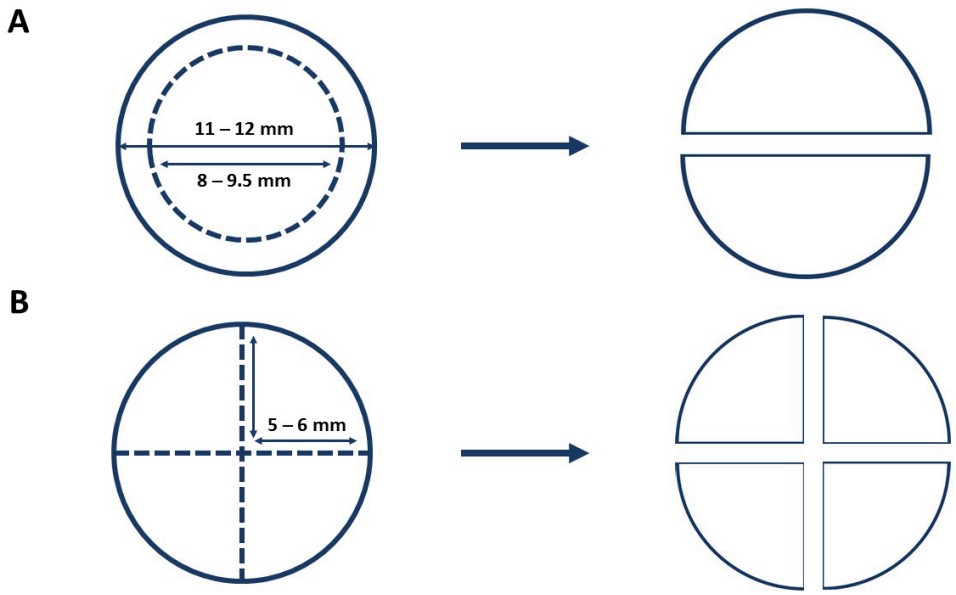

Figure 3: Hemi- and quarter-DMEK graft preparation. Schematic description of the preparation of hemi- and quarter-DMEK grafts. For hemi-DMEK grafts, a full-size endothelial graft is cut longitudinally in 2 semicircular halves with a 11-12 mm diameter, so to roughly cover the surface area of a standard DMEK graft of 8-9.5 mm diameter (A). Quarter-DMEK graft preparation requires the division of a full-size endothelial graft into 4 parts, with two longitudinal cuts, in order to obtain 4 quarter-DMEK grafts with a diameter of 5-6 mm (B).

A first cell migration study conducted on *in vitro* cultured Quarter-DMEK grafts showed an increase of cell migration between day 4 and day 6 of culture, but only from the cut edges. In contrast, there was no cell migration from the limbal round edge area, most likely due to the peripheral collagen fibers that impaired cell migration.[57] In order to stimulate migration of cells "trapped" in the collagen

structure, another *in vitro* cell migration study was conducted on cultured Quarter-DMEK graft, but this time the limbal edge of the Quarter-DMEK grafts was "customized" with small cuts and trephinations. Moreover, the grafts were cultured in a thermoresponsive gel, to facilitate the passage of nutrients of the culture media. While cell migration increased from the radial cut edges of the grafts, as a result of more favorable culture conditions, little to no cell migration was again observed from the limbal area, despite the modifications of the far periphery.[58] These results suggested that the peripheral area could be populated by a different type of cells that act as a cell reservoir, rather than displaying a migrative phenotype.

"Cell-based" treatment of corneal pathologies

To solve the global tissue scarcity of donor graft suitable for transplantation, "cell-based" approaches aimed to repair the damaged endothelial layer have been developed as an alternative to corneal endothelial keratoplasty. The main idea behind this approach is that hCEC could be isolated and cultivated *in vitro*, as hCEC have been shown to be able to proliferate if given the appropriate stimuli.[59] Finding out how to optimize *in vitro* hCEC proliferation has been a major target for many researchers in order to develop a reliable cell culture protocol by also taking into account the confounding aspect of lack of donor material from which to isolate and expand hCEC *in vitro*.

To bypass the dependency on scarce donor tissue, approaches have included the immortalization of CEC via viral transfection,[60,61] the disruption of the balance of cell cycle regulators,[62,63] the induction of genetic transformations that resulted in immortalized hCEC lines,[64] and optimization of hCEC culture conditions.[65-67] The first three approaches have serious impediments for a future clinical application, such as viral transformation, overexpression of exogenous genes, or aberrant karyotypes, since regulatory committees may describe them as dangerous and with a tangible possibility of inducing cancer in humans.

The possibility of using stem cells as a potential source of *in vitro* cultured CEC for clinical purposes has been described in the last years. Despite several protocols to derive hCEC from embryonic stem cells,[68-71] many concerns

regarding the use of stem cells, both on the ethical and safety level, appeared to have limited its suitability for further clinical application.[72]

Another potential source investigated for CEC production is the population of induced pluripotent stem cells (iPSC), a type of stem cells that can propagate indefinitely and be modified into any other cell type in the body, given the appropriate stimuli.[73] A practical application of this definition was the establishment of different protocols to derive CEC from iPSC, either from murine or human iPSC, under chemically defined conditions.[74,75] Nevertheless, the possibility to use iPSC as an effective source for cultured CEC is tempered by both biological factors, such as the still largely unknown conditions favoring the differentiation from human iPSC to CEC, and safety reasons, given the potential oncogenic risk linked to iPSC.[76,77] Moreover, like for the stem cells, in the absence of specific markers for hCEC it is difficult to properly distinguish the different cell types in culture.[77]

Mesenchymal stem cells (MSC) are nowadays easily retrieved from different human tissues, and in a theoretical way their use (similarly to other pluripotent cell types) seems more appropriate in comparison to primary hCEC, given their lower proliferative ability.[78] Both multipotent MSC and iPSC are derived from adult tissues, therefore fewer ethical issues arise, unlike embryonic stem cells. Moreover, a transplant of autologous MSC removes one of the side effects of allogenic grafts, namely immune-suppressive drugs to prevent rejection. MSC are a good candidate as a source for CEC production because, during human eye development, CEC differentiate from periocular mesenchymal cells.[79-81] In addition, these two cell types share the ability to express adhesion proteins such ZO1 and N-cadherin.[12] However, a definite protocol to establish an *in vitro* CEC population derived from pluripotent or stem cells has not been identified so far.

Despite the growing number of approaches to alternative sources for *in vitro* hCEC culture and expansion, the use of research-grade corneas still represents the preferred source, although donor characteristics and storage conditions of the donor material have an influence on the success rate of hCEC cultures.[67,82-84] The establishment of a reliable cell culture protocol for hCEC isolation and growth requires a fine-tuning of all the procedures involved: isolation of the corneal endothelium from donor corneas, enzymatic digestion of the corneal endothelium to obtain hCEC, seeding of the resulting hCEC cell suspension

using the most suitable combination of culture media and growth factors, and expansion and proliferation on appropriate substrates that mimic the *in vivo* condition (**Figure 4**).

One strategy to increase the cellular yield refers to the use of different areas of the human cornea. Trabecular meshwork from discarded corneoscleral rims has been described as a potential source for the establishment of hCEC cultures.[85,86] Moreover, a recent study conducted on cells from the transition zone (TZ), an area that includes the peripheral endothelium, the trabecular meshwork and the Schwalbe's line where the cells are increasingly being considered as adult stem cells.[87-90] Results showed that TZ cells could be potentially cultured *in vitro* when outgrown from human TZ explant, as they displayed proliferative capacity. An increase in the level of endothelial genes at the expense of the level of stem cells genes was also observed at later passages.[91] However, a different gene expression profile described for the hCEC residing in the peripheral areas of the corneal endothelium represents one of the major issues in elaborating a strategy for the establishment of an *in vitro* culture from these cells.[92] Indeed, it has been described that the cell population in the far periphery seems to be composed by different cell types, some of them expressing stem cell or fibroblastic markers.[93]

The current two strategies to deliver cultured hCEC onto the posterior corneal surface are corneal endothelial cell sheet transplantation and cell injection into the anterior chamber of the eye. Endothelial cell sheet transplantation requires a suitable cell carrier, which can have a natural origin or be a bioengineered matrix, and is still at a pre-clinical stage. On the other hand, cell injection obviates the need for a cell carrier, as the cultured CEC are injected directly into the patient's anterior chamber, and first clinical results are available since a couple of years.[94,95] Both strategies rely on the development of a robust and reproducible protocol for the *in vitro* culture and expansion of hCEC, that has to comply to good manufacturing practice (GMP) regulations in order to be suitable for clinical applications.

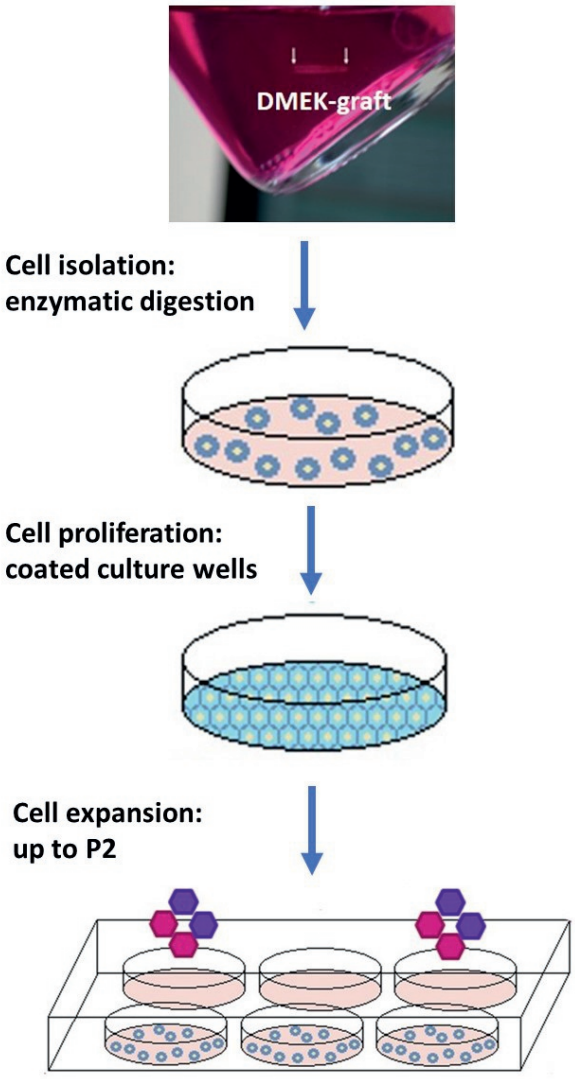

Figure 4: Human CEC isolation and culture protocol. *In vitro* hCEC culture starts from the peeling of the endothelial graft from a donor cornea. Tissue digestion allows the formation of single cells that are put in the condition to proliferate onto coated culture wells. Upon confluency, hCEC are passaged and expanded for several passages in order to obtain the suitable amount of cells for transplantation.

OUTLINE OF THE THESIS

Corneal transplantation still represents the elected method for the treatment of corneal endothelial pathologies. However, the worldwide shortage of donor corneas induced the exploration of approaches to use the donor tissue more efficiently or to be more independent from donor tissue. This thesis will illustrate the improvements of new strategies for cell-based corneal endothelial regeneration, alternative to corneal endothelial surgical transplantation, by bridging the gap between *in vitro* experiments and clinical models. In the studies described, we first address the establishment of a GMP-compliant protocol for *in vitro* hCEC culture for clinical application and then we focus on endothelial cell sheet transplantation, describing both *in vitro* and *in vivo* applications of expanded CEC-carriers constructs made by biocompatible materials.

In **Chapter 2**, a thorough overview of the current cell-based alternatives to corneal endothelial transplantation, as well as a focus on the challenges in the elaboration of a reliable protocol for *in vitro* hCEC culture and in the choice of the most suitable carrier for corneal endothelial cell sheet transplantation, is described.

In **Chapter 3**, we describe in detail a "modified" dual-media approach for *in vitro* hCEC isolation from single donor corneas derived from elderly donors to improve the success rate of establishing viable cell cultures.

In **Chapter 4**, the mechanism of action of a GMP-compliant collagenase is investigated to improve the cellular yield during cell isolation and to work towards a GMP-compliant hCEC culture protocol for clinical applications.

In **Chapter 5**, we describe the efficacy of bioengineered collagen carriers and human anterior lens capsule as substrates for pCEC expansion. The resulting cell-carrier sheets are tested in an *in vitro* surgery model as tissue-engineered alternative grafts for DMEK.

In **Chapter 6**, we use bioengineered collagen membranes, human anterior lens capsule and Descemet's membrane deprived of its cellular layers for hCEC expansion. An *in vitro* surgery model is used to compare the different cell-carrier constructs in comparison to the DMEK references model.

In **Chapter 7**, we describe the outcomes of *in vivo* testing of pCEC-HALC sheet transplantation in a pig model.

In **Chapter 8** and **9**, different and future outcomes are discussed.

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CHAPTER 2

New developments targeting corneal endothelial cell replacement.

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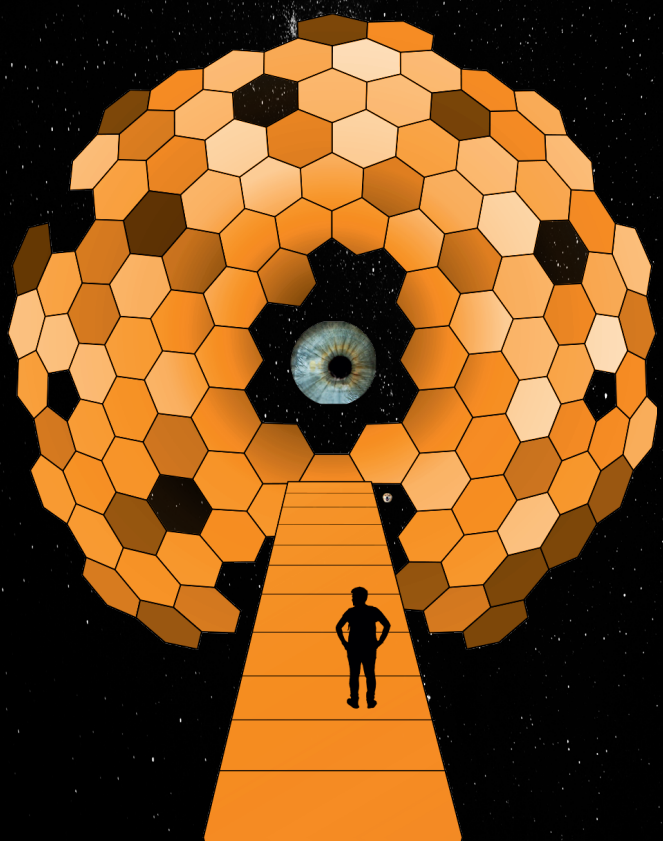

ABSTRACT

Corneal transplantation is currently the most effective treatment to restore corneal clarity in patients with endothelial disorders. Endothelial transplantation, either by Descemet Membrane Endothelial Keratoplasty (DMEK) or Descemet Stripping (Automated) Endothelial Keratoplasty (DS(A)EK), is a surgical approach that replaces diseased Descemet membrane and endothelium with tissue from a healthy donor eye. Its application, however, is limited by the availability of healthy donor tissue. To increase the pool of endothelial grafts, research has focused on developing new treatment options as alternatives to conventional corneal transplantation. These treatment options can be considered as either "surgery-based", i.e. tissue-efficient modifications of the currently endothelial keratoplasty techniques (e.g. Descemet Stripping Only (DSO)/Descemetorhexis Without Endothelial Keratoplasty (DWEK), hemi- and Quarter-DMEK), or "cell-based" approaches, which rely on *in vitro* expansion of human corneal endothelial cells (hCEC) (i.e. cultured corneal endothelial cell sheet transplantation and cell injection).

In this review, we will focus on the most recent developments in the field of the "cell-based" approaches. Starting with the description of aspects involved in the isolation of hCEC from donor tissue, we then describe the different natural and bioengineered carriers currently used in endothelial cell sheet transplantation, and finally we discuss the current "state of the art" in novel therapeutic approaches such as endothelial cell injection.

KEYWORDS: cornea, corneal endothelium, transplantation, tissue engineering.

INTRODUCTION

The corneal endothelium is a monolayer of hexagonal, tightly packed cells that marks the posterior part of the cornea. Human corneal endothelial cells (hCEC) are not thought to be capable of replication *in vivo*, being held in the G1 phase of the cell cycle.[1-3] As a result, hCEC have a restricted lifespan, meaning that their density and number decline naturally with age, with a decrease of ~0.6% per year from about 3500 – 4000 cells/mm^2 at birth to 2300 cells/mm^2 by the age of 85.[4-6] When hCEC are damaged, a cascade of events are initiated to repair the defect. Adjacent cells migrate centripetally, form new tight junctions and finally restoring the cellular pump function. During this process, the migrating cells remodel from an irregular endothelial cell to a more hexagonal pattern, though with fewer cells/mm^2.[7] The minimum density reported for a corneal endothelium to remain functional is thought to be approximately 500 cells/mm^2.[8]

Two of the most common pathologies of the corneal endothelium are Fuchs endothelial corneal dystrophy (FECD)[9] and bullous keratopathy.[10] These pathologies are commonly treated by corneal transplantation to restore corneal clarity in patients with endothelial disorders.[8] Descemet membrane endothelial keratoplasty (DMEK) is the most selective corneal transplantation technique, where the patient's Descemet membrane (DM) and damaged endothelium are removed and replaced with the same layers, from a donor eye.[11,12] However, its application is limited by global shortage of high-quality donor tissue, with only one donor available for every 70 patients in need of a transplant.[13] Different strategies have been applied in the past decades to solve the tissue shortage and these may be broadly separated into two approaches: the surgical and the "cell-based" approach.

Surgical approaches to target tissue shortage include Descemet Stripping Only (DSO)/Descemetorhexis Without Endothelial Keratoplasty (DWEK) and Quarter-DMEK technique, which were developed for eyes with central FECD. With DSO/DWEK, the diseased tissue is removed without replacement by donor tissue. The stripped region is re-populated by the migration of the peripheral endothelial cells that can restore corneal clarity, though with varying rates of success.[14-16] Quarter-DMEK resembles standard DMEK, but the donor

DM-endothelial cell sheet is divided into four grafts allowing a much more efficient use of donor tissue.[17,18] Clinical results up to 2-year follow-up for Quarter-DMEK show visual rehabilitation comparable to standard DMEK, but a higher endothelial cell density (ECD) decrease than standard DMEK.[19,20]

The "cell-based" approaches rely on the *in vitro* expansion of isolated primary hCEC (**Figure 1**), as hCEC have been shown to be able to proliferate *in vitro* if given the appropriate stimuli.[21] Currently, the main strategies to deliver hCEC onto the posterior corneal surface are cultured endothelial cell sheet transplantation and cell injection into the anterior chamber. Endothelial cell sheet transplantation requires a suitable cell carrier, which can have a natural origin or be a bioengineered matrix, and is currently still in a preclinical stage. Cell injection therapy, on the other hand, obviates the need for a cell carrier, as the cultured hCEC are injected directly into the patient's anterior chamber. For this approach, first clinical results have been reported.[22,23]

In this review, we focus on the two "cell-based" approaches, cell sheet transplantation and cell injection, by covering the entire approach from *in vitro* growth of primary hCEC over potential carrier for cell sheet transplantation to considerations for cell injection therapy to first pre-clinical and clinical result.

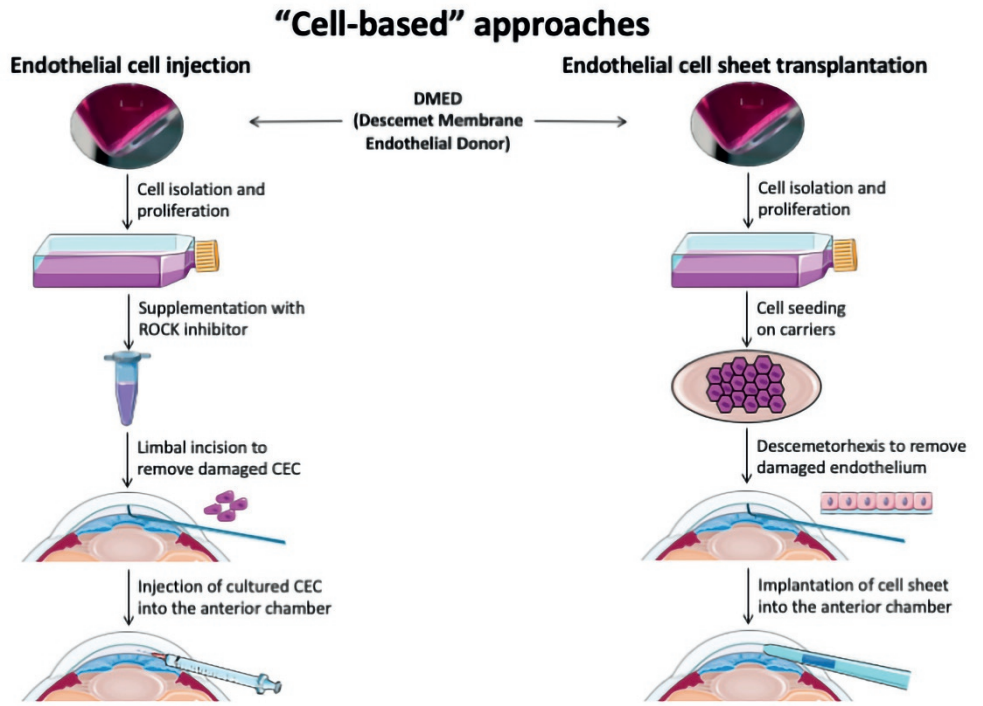

Figure 1: "Cell-based" approaches. Schematic representation of the two "cell-based" approaches described in this review: the endothelial cell injection approach and endothelial cell sheet transplantation approach. They both rely on the availability of a donor tissue (DMED = Descemet Membrane Endothelial Donor), from which hCEC will be isolated and subsequently cultured and expanded in vitro. At this point, for the cell injection approach, suspended hCEC will be supplemented with ROCK inhibitor and injected into the anterior chamber of the patient, after having removed the damaged endothelial tissue from the eye of the patient. For the endothelial cell sheet transplantation approach, cultured hCEC are passaged upon confluency, suspended in culture medium, seeded on the desired carriers, and then transplanted following a DMEK-like surgical protocol, upon descemethorexis to remove the damaged endothelial layer.

IN VITRO CULTURE AND EXPANSION OF CORNEAL ENDOTHELIAL CELLS

Although hCEC have limited proliferative capacity *in vivo*, they do have the ability to proliferate *in vitro*. After the first reported successful *in vitro* expansion of hCEC in 1965,[24] a plethora of protocols describing the isolation and *in vitro* growth of hCEC have been published.[8,23,25-49] The abundance of protocols is mainly due to the challenges inherent in culturing and expanding hCEC *in vitro*, and so far, no protocol appears to be superior regarding the consistent expansion of hCEC. Successfully establishing a robust and reproducible cell culture protocol for hCEC isolation and expansion requires a fine-tuning of all the procedures involved: selection of suitable donor tissue, peeling of the corneal endothelium and DM from donor corneas, enzymatic digestion to isolate the hCEC, seeding of the resulting cell suspension using a combination of culture media and growth factors, and expansion and proliferation on appropriate substrates that mimic the *in vivo* conditions. It has been shown that the induced loss of cell contacts and the supplement of the culture medium with selected growth factors boost hCEC growth.[50] Multiple extracellular signals, however, can activate critical intracellular pathways and induce endothelial-to-mesenchymal transition (EMT) of hCEC, where the cells acquire a fibroblast-like phenotype thereby losing their morphological features and, most importantly, their function.[8,34,40,50,51] Therefore, a lot of effort has been put in designing the appropriate protocols for hCEC isolation, expansion and propagation with a focus on maintaining hCEC morphology and function.

Sources for corneal endothelial cell culture

Primary hCEC isolated from cadaver donor corneas, stem cells, and cell lines have all been used as sources for corneal endothelial cell culture.[52] The main sources of primary hCEC are human cadaveric donors, however, donor characteristics such as cell density,[41] cause of death, previous surgery in the donor eye,[53] overall health of the donor, and tissue storage time can have a significant impact on the culture success rate.[54,55] Donor age can also play a role[42] with a

usually lower proliferation capacity for hCEC derived from older donors.[25,29,33,34,45,47,56-61]

It has also been reported that hCEC from the corneal periphery have a higher proliferation capacity than hCEC from the corneal center,[30,55,57,58,62,63] though another study showed no difference in the replicative capacity between the peripheral and central areas of the cornea.[55] Attempts to overcome the lack of donor material from which to isolate and expand hCEC *in vitro*, have included the immortalization of CEC via viral transfection,[64,65] the disruption of the balance of cell cycle regulators,[66,67] and the development of genetic transformations that resulted in immortalized hCEC lines.[68]

Stem cells used for hCEC culture include organ-specific adult stem cells obtained from adipose tissue, umbilical cord blood or bone marrow,[69-71] directed differentiation competent embryonic stem (ES) cells,[72] induced pluripotent stem (iPS) cells,[73] and hCEC precursors.[74] For the latter, it has been suggested that existent stem-like progenitors of hCEC may retain more proliferative capacity compared to terminally differentiated hCEC.[75] It is thought that such hCEC progenitors could be present in the area that separates the peripheral endothelium from the anterior part of the trabecular meshwork, and they might act as a cell supply activated in the event of a wound healing.[35,37,74,76-81] However, these cells have been hard to identify because of a lack of suitable markers and their existence is still debated.[77] In addition, the amount of stem cells found in this compartment of the cornea may be very low and could require an extended *ex vivo* culture to generate sufficient cells for transplantation.[82]

Methods of isolation of corneal endothelial tissue

Currently, the most used protocol for cell isolation consists of a two-step peel-and-digest method (**Figure 2**).[34,35,38,39,42,43,45,47,54,60,83-93] The Descemet Membrane Endothelial Donor (DMED) is first peeled from the underlying stroma of the donor cornea, followed by dissociation of cell junctions to separate the hCEC from the membrane. In respect to the method used to disrupt cell junctions, the tissue digestion strategies may be classified as enzymatic and non-enzymatic.[82] The enzymatic digestion procedures are based on enzymatic treatments such as

collagenase, trypsin, or dispase, while the non-enzymatic tissue digestion uses ethylenediamine tetraacetic acid (EDTA) to release cell-cell junctions.[60] In enzymatic digestion, several approaches have been proposed,[26,51] including the use of collagenase A as this enzyme induces a careful, selective reduction of the intercellular matrix with minimal damage to cell membranes and the ability of hCEC to expand.[35,38,74] Another enzymatic digestion approach uses trypsin, which has been used successfully to cultivate and expand hCEC on bovine extracellular matrices.[29] Nevertheless, trypsin mainly acts on the intracellular mucoproteins, thus affecting the cell membrane and is typically used in passaging monolayer cultures. For a more effective action during cell isolation, trypsin is often used in combination with EDTA, which disrupts lateral cell contacts thus separating cells from each other as well as from the culture surface without negative effects on cell viability.[94] Liberase and dispase have also been shown to be useful in the isolation of CEC from corneal endothelial tissue.[49,83]

The non-enzymatic method is based on the mechanism of action of EDTA to release cell junctions while it enhances cell division upon exposure to mitogens.[54,55,62,70,94] Other approaches, usually applied in the beginning of hCEC isolation, include hCEC scraping from the DM,[27] enzymatic digestion of hCEC on the cornea,[26] a combination of enzymatic digestion of hCEC on the cornea and scraping,[55] and explant culture.[28,33,42,95-99] Furthermore, recent efforts have been made to isolate a purer population of hCEC with high proliferative capacity by density-gradient centrifugation to eliminate senescent cells.[100] However, because of different issues of both enzymatic and non-enzymatic methods alone (contamination with other cellular types and decrease of cellular yield, respectively), the preferred method to date is a combined treatment that includes collagenase, to derive hCEC aggregates from the tissue, and incubation for short time with trypsin/EDTA to produce less cell damage.[82]

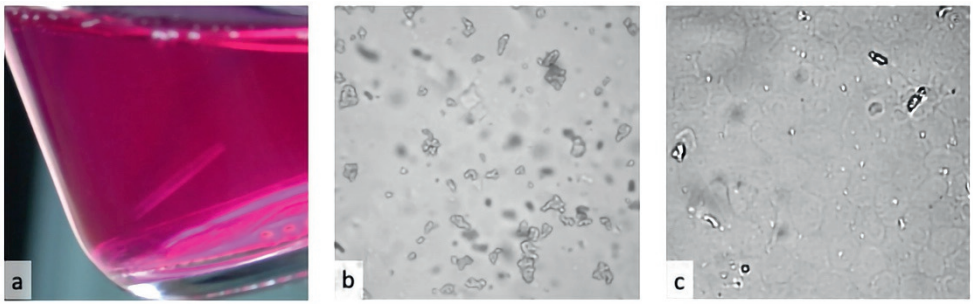

Figure 2: The "peel-and-digest" method. Series of images illustrating the 2-step, "peel-and-digest" method applied for the hCEC isolation from donor tissue. After the peeling of the DMED from the donor cornea, the tissue is stored accordingly in the eye bank (a). Upon hCEC isolation, DM-EC sheet is exposed to collagenase digestion for 4-6 hours to disrupt the cell junctions and to obtain smaller fragments, which will be further dislodged in single cell suspension by flushing and centrifugation (b). After 2 weeks in culture, hCEC were able to form a uniform monolayer of tightly packed cells, which retained their characteristic hexagonal morphology (c).

Culture media and growth factors

Many hCEC culture media have been described with a combination of different base media (e.g. M199, L-valine-free IF, F99, Ham's F12 + M199, Ham's F12+DMEM (SHEM), MEM, Endothelial growth medium, DMEM, OPTIMEM-I), essential and non-essential amino acids (e.g. glutamine), sera (e.g. bovine, calf, horse, human), antibiotics (e.g. penicillin, streptomycin, gentamycin, amphotericin B, fungizone, doxycycline), growth factors or a combination thereof (e.g. basic fibroblast growth factor (bFGF), insulin, nerve growth factor, bovine pituitary extract, epidermal growth factor, transferrin), vitamins (e.g. ascorbic acid, l-ascorbic acid 2-phosphate) and other components (e.g. chondroitin sulphate, calcium chloride, DMSO, hydrocortisone, cholera toxin, selenium, sodium selenite, human lipids, RPMI-1640 multiple vitamin solution, type-I collagenase, B-27, Rho-associated protein kinase (ROCK) - inhibitor, cysteine, TGFβ-inhibitor).[28,29,33,34,37-39,41,43,45,47,51,52,55,56,60,62,84-88,91,92,95,98,99]

However, while none of these media may be superior in the reproducible cultivation and expansion of hCEC *in vitro*, the use of a so-called "dual-media" approach has gained popularity.[21,42,43,89,101,102] Peh et al. were the first to describe

a dual-media approach, in which isolated hCEC were propagated using a culture system that included a proliferative medium, rich in growth factors, and a serum-supplemented culture medium, without growth factors, in order to avoid epithelial-mesenchymal transition (EMT). A modified dual-media approach has also been described to increase the rate of obtaining successful cultures from elderly donor corneas.[47]

In addition, several studies reported the use of conditioned medium from other cell types to promote hCEC growth. These include conditioned medium from murine embryonic stem cells (mESC),[39,98] human bone marrow-derived mesenchymal stem cells (hBM-MSC),[39] and human amniotic fluid (hAF).[103] The first two treatments showed to be effective in hCEC proliferation and motility by predominantly acting on the cell cycle level, while hAF-containing culture medium stimulated hCEC growth. While conditioned medium from mESC is not suitable for clinical application due to the presence of a xenoantigen for human cells, condition media derived from both hBM-MSC and hAF can be used in the clinical setting. Condition medium from hAF is cheaper and more readily available than other growth factors, however the potential interdonor variability does not make this product fully suitable for clinical applications.

Preventing endothelial-to-mesenchymal transition of hCEC during culture

Preventing EMT is one of the most challenging issues in hCEC culture.[8,34,40,50,51] During EMT, hCEC loosen their specific markers and adopt mesenchymal characteristics. While EMT actually plays an important role in normal physiological processes such as wound healing, it also interferes with pathological processes such as fibrosis.[50] The onset of EMT can be identified by typical events such as: disruption of cell-cell junctions, loss of cell polarity, modifications in cell shape and cytoskeletal organization, increment in the secretion of ECM proteins and genotypical changes (**Figure 3**).[50] This loss of endothelial phenotype represents a big limit for the use of cultured hCEC in tissue engineering applied to corneal regeneration.[40,50,104-110] Thus, in order for cultured hCEC to be applied in clinical practice, EMT must be suppressed during the culture process.[50]

Several strategies have been proposed to block EMT. As *in vitro* hCEC culture and expansion requires steps like cell isolation and passaging that inevitably cause the dissociation of cell junctions, culture conditions should be such that cell confluency and polarity are gained as fast as possible.[50] This can be accomplished by steps as simple as increasing cell seeding density.[41] During EMT, reorganization of the cytoskeleton can be visualized by the presence of α-smooth muscle actin (α-SMA).[104,111,112] The expression of α-SMA by hCEC has been shown to be promoted by transforming growth factor beta (TGF-β), a cytokine known to induce EMT.[40,104]

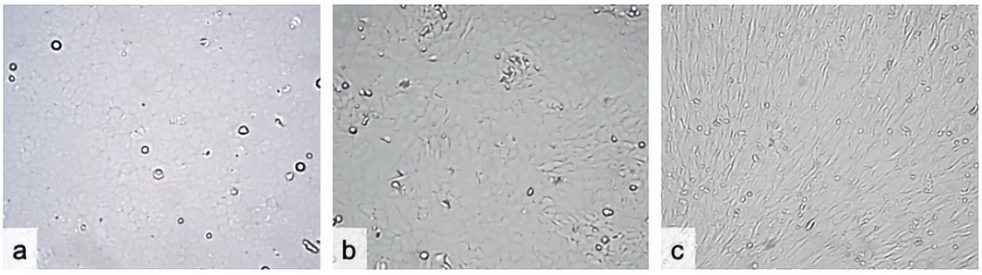

Figure 3: EMT transition in cultured hCEC. Light microscopy images showing the transition from a successful hCEC culture to a fibroblastic culture following EMT. After enzymatic digestion, hCEC are expanded and form a confluent monolayer of small, hexagonal cells (a). Due to characteristic events such as changes in cellular gene expression and phenotype and loss of cell-cell junctions, cultured hCEC could undergo EMT, facing different phases from a culture of mixed phenotype (b) to a full fibroblast-like culture (c). This process is particularly disadvantageous because it limits the use of cultured hCEC for further purposes in tissue engineering.

In normal cells, TGF-β arrests the cell cycle at the G1 stage, inhibiting proliferation and inducing differentiation. Studies have shown that hCEC are amongst the cells most sensitive to stimulation by TGF-β, with TGF-β having both pro-migratory and anti-proliferative effects on hCEC.[105] In addition, blocking the TGF-β(2) receptor in proliferating hCEC by anti-TGF-β inhibitor SB-431542 allows expansion and proliferation, by avoiding EMT and uncontrolled growth of other cell types.[40] L-ascorbic acid 2-phosphate (Asc-2P), a phosphate derivative of vitamin C,[113] acts as an antioxidant and a stimulator of

hepatocyte growth factor (HGF) production previously known for stimulation of the barrier function in endothelial cell monolayers[114] and the proliferation and replication of a number of cell types.[115,116] Adding Asc-2P to hCEC culture media can also aid in cell growth, proliferation, and in delaying EMT.[87,117]

Additionally, cells in which EMT is induced can increase the production of ECM proteins, such as collagens, cadherins and fibronectins.[40] *In vitro* models of endothelial diseases were used to find out several factors involved in EMT that induced atypical ECM secretion and fibrosis by hCEC, including FGF-2.[50] Paradoxically, FGF-2 is often used to stimulate hCEC proliferation *in vitro*, despite the risk of inducing EMT of hCEC. The fine balance between FGF-2 ability to either induce proliferation or EMT in cultured hCEC is currently under investigation.[50]

Coatings

Descemet membrane is the basement membrane secreted by the corneal endothelium and includes many proteins, such as fibronectin, laminin, collagen type IV and VIII and proteoglycans,[118-123] and considerable developments have been done towards the design of a resembling native ECM environment for hCEC growth and proliferation.[124-133]

Although hCEC are capable of attaching to and expanding on the tissue culture plates,[42,60,85,98] the use of coating materials has shown superior results. Tested types of coating materials for hCEC expansion include varieties of collagen,[31,42,51,83,85,87,92,96,99,134-138] fibronectin,[27,29,42,51,85,99,131,137] laminin,[51,90,99,131] and gelatin.[27,139-142] Also mixtures of different components have been used,[26,29,33,51,95-97,99,143] of which the mixture of fibronectin, collagen, and albumin is the most widely used coating (FNC coating mix®).[34,39,42,43,45,47,54,88,93,131]

It has been reported that FNC-coated culture plates improve the spreading of hCEC in culture and also reduce cell loss after rinsing.[36] Moreover, hCEC cultures grown on FNC-coated plates showed a higher success rate in terms of cell confluence and morphology compared to cultures grown on fibronectin-coated plates, collagen IV-coated plates, and uncoated plates.[42]

Laminin is another major component of the basement membrane, and is responsible for the regulation of cell migration, proliferation and differentiation.[144,145] Laminin isoform laminin-5 (LM-5) has been shown to have a positive effect on hCEC adhesion and migration.[99] Some other isoforms of laminin (laminin-511 and laminin-521), also expressed in DM, are good substrate candidates for *in vitro* hCEC culture, helping to increase cell adhesion and proliferation.[90] Moreover, the inclusion of laminin E8 fragments (truncated proteins that include the active binding site)[146] help cultured hCEC retain a better endothelial cell morphology compared to hCEC cultured on FNC coating mix.[90]

Overall, however, the biological activity of hCEC on these coatings, even among studies using the same coating material, varies. This might be explained by the type of coating affecting hCEC adhesion, morphology, proliferation, and function.[52] In addition, the variation among studies using the same coating might be explained by differences in the applied cell culture protocol.[52] Nevertheless, the translation of these coatings to the clinical application is still doubtful since they are derived from animals which may bare the risk of pathogen transfer. The development of a recombinant form of laminin E8, however, offers the possibility of a xeno-free, good manufacturing practice (GMP)-compliant substrate.[146] An alternative xeno-free substrate could be a pericellular matrix of decidua-derived mesenchymal cells (PCM-DM). This matrix has already been tested, with good results, as a substrate for human ES cells,[147] iPS cells,[148,149] and hCEC.[137]

CULTURED ENDOTHELIAL CELL SHEET TRANSPLANTATION

Human CEC, successfully cultured and expanded *in vitro,* form intercellular junctions creating a cell sheet. A cell sheet, however, is too fragile to be handled surgically and requires support. To that end, substrates or carriers that provide mechanical support during transplantation of *in vitro*-cultured hCEC have been developed and tested. An ideal carrier should mimic the basic characteristics of the DM and therefore be transparent, permeable, not too thin to provide sufficient mechanical strength (as the elastic modulus could have an impact on cell phenotype),[132] flexible to adjust to the corneal curvature, biocompatible, promoting hCEC-carrier interactions, and allowing interaction between the cultured hCEC layer and the recipient stroma in terms of exchange of nutrients and small molecules, and finally, be easily reproducible.[52] Potential carriers, that can either be natural tissue materials such as amniotic membrane, human anterior lens capsules (HALC), and decellularized DM or stroma,[46,48,68,83,84,136,143,150-153] or polymeric materials (natural and synthetic), have been explored as carriers to facilitate hCEC transplantation.[31,85,86,88,131,132,142,154-159]

Natural tissue carriers

Denuded DM and Devitalized Stroma

As DM is part of endothelial grafts, and given that devitalized corneas or denuded DM supply the desired shape, mechanical strength and transparency, they have been widely tested as carriers for hCEC.[52,60,95,130,131,143,150,152,160-162] The endothelial cells from donor DM can be removed by enzymatic treatment, in most cases with a combination of trypsin and EDTA (**Figure 4 c, d**), or via mechanical treatment or by applying several freeze/thaw cycles, to obtain a denuded DM.[95,150,160-165]

Mimura et al. showed the feasibility of corneal reconstruction with cultured hCEC seeded on denuded DM and transplanted in nude rats.[150] Up to one month after transplantation, hCEC functioned well and maintained corneal transparency. Another study, that used devitalized posterior corneal stromal

lamellae as a hCEC substrate, showed that the graft had intact barrier function and the hCEC expressed typical markers.[152] Recently, in a rabbit model of bullous keratopathy, Peh et al. showed that hCEC seeded onto denuded DM or stroma were able to induce corneal clearance.[46] However, when using devitalized corneal stroma, resident viable keratocytes may cause fibroblastic contamination.[52]

Using an *in vitro* surgery model (**Figure 5 e-h**), hCEC-denuded DM constructs behaved similarly to the DMEK reference model. The constructs, however, displayed a 'reversed' rolling with the hCEC on the inside.[166] In addition, this study showed that denuded DM derived from elderly donors show a highly structured surface due to imprints left by the removed cells (**Figure 4 c, d**). This structured surface seemed to be responsible for the impaired morphology of the cultured cells on these carriers.

Human anterior lens capsule

The human anterior lens capsule (HALC) is a transparent membrane that is mainly composed of interacting networks of laminin[167-170] and collagen type IV,[168,171,172] the latter being HALC's most prominent protein,[172] forming up to 40% of its weight.[173] In addition to its composition, the physical characteristics of HALC are also very similar to the DM in terms of thickness, elasticity and transparency.[174,175]

HALC has been investigated as a scaffold for culturing and transplanting different ocular cells,[176-180] including hCEC.[84,136,166] For these purposes, HALC can be either manually isolated from donor eyes followed by an enzymatic treatment to remove cellular layers from the surface (**Figure 4 a, b**), or can be obtained during cataract surgery, limiting on one side the available diameter to 6 mm approximately but allowing the isolation from the patients themselves.[84,136,180] It has been shown that hCEC density and morphology when grown on HALC resembled those of healthy corneas.[84] The surgical potential of CEC-HALC carrier constructs was tested in an *in vitro* surgery model (**Figure 5 a-d**).[166,180] Upon *in vitro* surgery, hCEC-HALC constructs behaved in a manner similar to the DMEK reference model. Interestingly, the hCEC-HALC

constructs exhibited a 'reversed' rolling property, with the endothelial cells on the inside. First *in vivo* tests in an animal model of CEC-HALC constructs were not successful due to the choice of the animal model system. The Goettingen mini-pigs used in that study showed an enhanced retrocorneal membrane formation limiting the information to be obtained on the CEC-HALC constructs.[181]

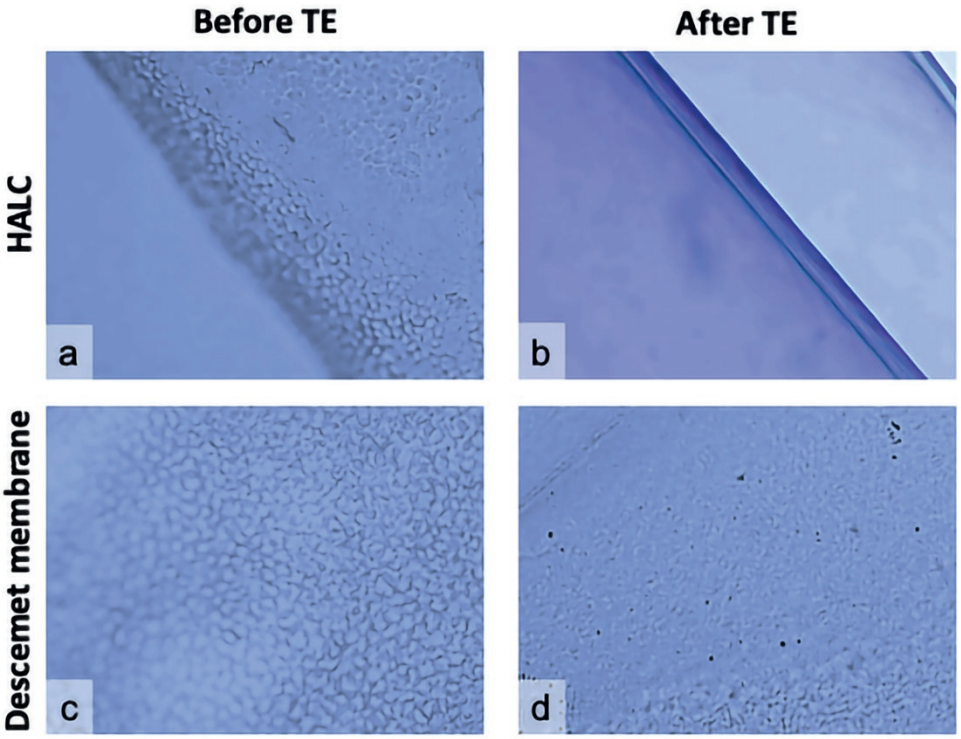

Figure 4: HALC and "denuded" Descemet membrane preparation. To use human anterior lens capsule (HALC) and Descemet membrane as a carrier for hCEC sheet transplantation, cellular layers had to be removed from both sides of the substrates. When HALC was subjected to an enzymatic treatment with Trypsin/EDTA (TE) solution for 15 minutes, smooth sides were obtained (a, b). On the other hand, the treatment of Descemet membrane with TE solution was efficient to remove the cell layer, but the surface showed a structured pattern, most likely left by the removed cells (c, d).

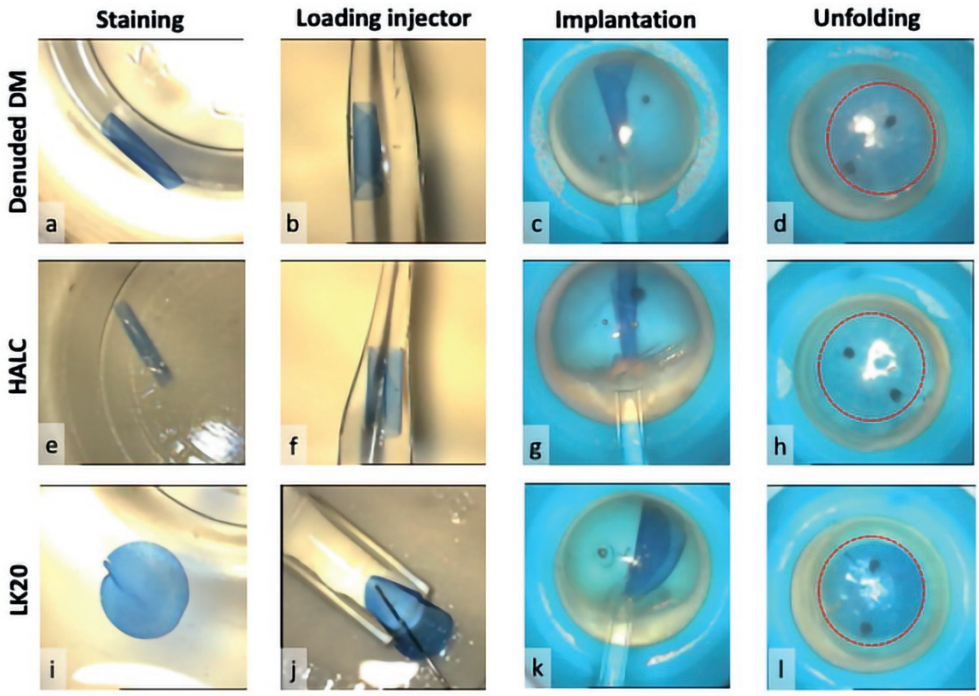

Figure 5: *In vitro* surgeries with hCEC-carrier constructs for endothelial cell sheet transplantation. *In vitro* surgeries were performed to test the feasibility of hCEC-carrier constructs as potential alternatives to corneal grafts. The analyzed carriers were Descemet membrane (DM) from which the endothelial layer was removed (denuded DM), human anterior lens capsule (HALC), and collagen I-based carrier of 20 µm of thickness (LK20). The constructs were implanted using a DMEK surgical technique into an anterior remnant without endothelium mounted onto an artificial anterior chamber and several parameters were evaluated, such as staining with hypotonic Trypan Blue solution 0.04% (a, e, i), loading into the glass injector (b, f, j), implantation into the artificial chamber (c, g, k), and unfolding (d, h, l).

Amniotic membrane

Amniotic membrane is composed of collagen type IV[182] and has been widely used for surgical purposes.[183] It was the first successful natural carrier for ocular cell culture and transplantation[184] and is known to have anti-inflammatory[185] and non-immunogenic[186] properties. It has been demonstrated by Ishino et al. that ECD and morphology of hCEC cultured on amniotic membrane were similar to those of normal corneas, and such cultured cells were also functional *in vivo*.[83] After transplantation of the hCEC-amniotic membrane constructs into rabbit

eyes, corneal edema was observed after seven days.[83] Fan et al. showed that hCEC cultured on amniotic membrane retained normal morphology and marker expression. Moreover, after transplantation in a cat model where the cornea was denuded of endothelium and DM, corneas remained transparent throughout the monitoring period.[68] However, while amniotic membrane is biocompatible along with anti-inflammatory and non-immunogenic properties, potential disadvantages are the lack of transparency, and the time-consuming preparation time.[52,187]

Bioengineered carriers

To reduce dependency on donor tissue, there is an increasing interest in tissue-engineering of (bio)synthetic cell carriers,[140,142,188,189] though the issues of biotoxicity and bio-incompatibility make this approach more challenging.[190] Ideally, the carrier should mimic the natural basement membrane as much as possible in terms of composition, architectural durability, biocompatibility with cultured CEC, transparency and compliance for clinical application.[159] Several biomaterials, both natural and synthetic polymers, have been investigated.[31,85,86,88,131,132,142,154-159] Natural polymers, such as collagen, gelatin, and silk are usually biocompatible, though synthetic polymers guarantee a fine customization of the desired properties.

Collagen-based carriers

Collagen is the most abundant component of ECM in most tissues.[159] While it is easy to produce, cheap to derive from animal sources, and compatible with corneal cells, by itself it is not sufficiently rigid to handle surgically. Crosslinking the collagen improves its mechanical strength and chemical stability, and this can be achieved by chemical, physical, and enzymatic procedures.[159] One concern about the use of crosslinked collagen is the generation of toxic residues.[191]

Culturing hCEC on collagen sheets is not new, as studies in this area date back to the 1980s,[192] and showed promising results in animal studies.[92,96,193] Mimura et al. demonstrated that cultured hCEC, transplanted via a crosslinked collagen-1

sheet, can maintain corneal dehydration in a rabbit model.[96] When hCEC were cultured on collagen-vitrigel, they displayed a strong expression of ZO-1 and Na^+/K^+-ATPase, and reduced corneal thickness and improved corneal transparency in a rabbit model.[193] In another rabbit study, transplanted hCEC-collagen hydrogel sheets maintained a high cell density and N-cadherin, ZO-1, and actin-filament displayed a similar expression pattern as in native hCEC.[92]

While these studies reveal that cells can maintain their morphology and function on the collagen, the flexibility and thickness are also important to consider. Collagen type I-based carriers, of different thickness, have been tested by *in vitro* surgery. A carrier of 20 μm thickness (LK20) exhibited good biocompatibility, however, during *in vitro* surgery, the hCEC-LK20 constructs displayed poorer adherence to the posterior stroma than the other carriers tested (**Figure 5**).[166] A carrier of 100 μm thickness of the same material was found too rigid for the intended surgical procedure.[180]

Modifying the collagen to create collagen-polymer composites (chitosan, chondroitin sulphate) has been reported to further increase the mechanical support and resistance to enzymatic degradation.[159] Plastic compressed collagen type I, termed Real Architecture For 3D Tissues (RAFT) may offer better mechanical properties when compared to crosslinked collagen.[88] The formation of a confluent monolayer of hCEC which expressed ZO-1 and Na^+/K^+-ATPase on these carriers was confirmed by scanning and electron microscopy imaging.

Although the deep understanding of collagen-based sheets as a potential carrier for transplantation, the translation to clinical application still faces several challenges. Collagen is a material derived from animal origin and this raises questions in terms of immunogenicity and interspecies transmission of diseases.[194] Moreover, the extraction and purification procedures make collagen very expensive for large amounts of material needed for clinical applications and such procedures can vary from company to company, resulting in unwanted batch-tobatch variations.[195] Clinical-grade carriers should guarantee cell adherence and pumping function for the cells seeded on the carriers (**Figure 6**). Several research groups have tried to synthetize recombinant human collagen, but the low yield and the scarce post-translational modifications still represent unsolved issues.[196]

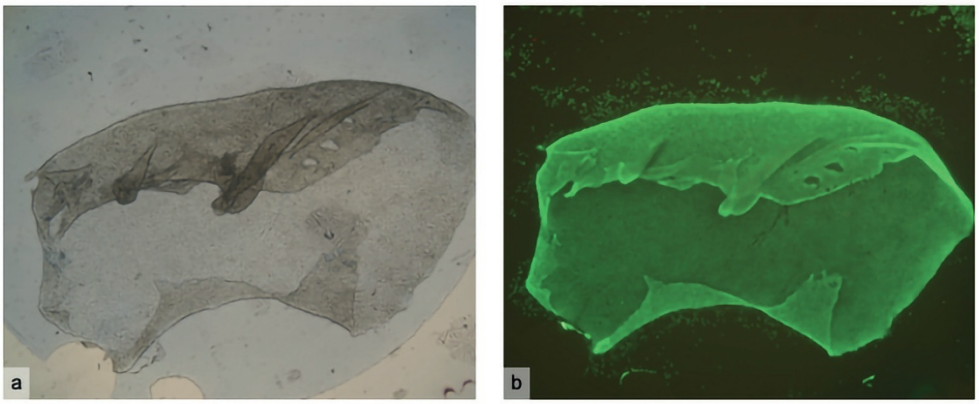

Figure 6: Cultured hCEC adherence on collagen-based carriers. After hCEC seeding on a collagen-based carrier, the cell-carrier construct was tested for its feasibility as an alternative to endothelial graft upon *in vitro* surgery. After the surgery, the cell layer detached almost completely from the carrier below (a), although the Calcein-AM staining confirmed that the seeded hCEC were viable (b). These results show that cell adherence still represents a parameter that should not be taken for granted in the development of a firm interaction between cultured hCEC and biocompatible carriers.

Gelatin-based hydrogels

Gelatin is a commonly obtainable natural material originated from the hydrolysis of collagen,[159] and when crosslinked, gelatin-based cell carriers can be used for tissue-engineering.[159] Watanabe et al., showed that primary hCEC seeded on hydrothermally crosslinked gelatin could form a confluent monolayer and showed normal marker expression.[142] Adding heparin during chemical crosslinking produced a gelatin carrier flexible enough to be folded, and that maintains morphology and pump function of hCEC seeded onto it.[156] The use of semisynthetic gelatin methacrylate registered an increase in tissue engineering because of its biocompatibility and adaptable physical characteristics,[159] and has been shown to support hCEC *in vitro* as well as after transplantation into a rabbit model.[158]

Chitosan

Chitosan is a biomimetic polysaccharide derived from the deacetylation of chitin.[188,189] Blended membranes made of chitosan, gelatin and chondroitin sulfate have displayed biomechanical properties (e.g. transparency, ion and glucose permeability) comparable to the human cornea. During *in vivo* testing in rabbit eyes, the CEC-blended membrane constructs showed steady degradation of the membrane and this did not influence the structure and the curvature of the cornea.[197] A keratin-chitosan membrane has also been shown to support CEC attachment and growth while maintaining cell morphology and marker expression.[198] In addition to providing a carrier for hCEC, the incorporating chitosan can improve the optical transparency and mechanical support of collagen membranes.[197] In another study, a chitosan-based membrane was constructed of hydroxypropyl chitosan, gelatin, and chondroitin sulphate.[199] Although optical transparency was comparable to the natural human cornea and cultured rabbit corneal endothelial cells formed a monolayer on the blend membrane, mild signs of inflammation were observed *in vivo*.[199]

Silk fibroin

Silk fibroin has been extensively used in regenerative medicine because of its optical properties, non-immunogenic response, flexible degradation standards and mechanical characteristics.[159,200] Silk fibroin membranes have been shown to support the formation of a confluent hCEC monolayer.[86,200] These hCEC-membrane constructs, with cells showing normal morphology and expression of characteristic markers, were trialed in a rabbit model.[200] Corneal transparency was maintained up to 6 weeks after transplantation without inflammatory reactions and the silk fibroin films remained optically transparent.[200]

Thermoresponsive polymers

Thermoresponsive polymers, such as poly(N-isopropylacrylamide) (PNIPAAm) and copolymers based on it, offer the advantage of thermally modulating their hyrophobic and hydrophilic properties which facilitates the detachment of cell

sheets without enzymatic digestion.[159] The use of a thermoresponsive polymer was first described by Hsiue et al.[140] In a temperature-mediated process, hCEC were seeded and cultured on hydrophobic poly(N-isopropylacrylamide) (PNIPAA)m-grafted surfaces at 37˚C. Once the culture temperature was lowered to 20˚C, the resulting hCEC sheet detached from the now hydrophilic PNIPAAm-grafted surfaces and were implanted in rabbit eyes together with an adhesive gelatin hydrogel disc. After swelling and biodegradation of the gelatin hydrogel disc, the transplanted hCEC sheet was fully attached to the posterior stroma and the corneas were clear, with near normal corneal thickness within two weeks.[140] While thermoresponsive polymers may offer interesting properties as a potential carrier, more detailed analysis on how the temperature change effects hCEC functionality is still lacking.

Other synthetic carriers

Recently, biodegradable electrospun poly(glycerol sebacate)-poly(ε-caprolactone) blend nanofibrous,[201] and poly(lactic-co-glycolic acid) scaffolds[202] were tested *in vitro* as possible carriers for hCEC by showing a monolayer of cultured hCEC with normal morphology. In addition, a hybrid carrier was constructed with a surface-initiated assembly technique: this hybrid was composed of basement membrane proteins (collagen IV and laminin), supported by a collagen-1 gel to mimic DM and a layer of stroma with a consistent thickness of 10μm, therefore similar in composition to a DSEK-graft but more similar to a DMEK-graft in terms of thickness.[203] Human CEC seeded on this carrier formed a monolayer and expressed continuous ZO-1 at their borders, showing that this technique could generate a biocompatible membrane with biomechanical properties similar to those of a native basement membrane and therefore suitable as a bioengineered corneal endothelial graft.

CELL INJECTION THERAPY

First approaches to cell injection therapy

Cultured CEC can also be directly injected into the anterior chamber of the eye, avoiding the need for a carrier entirely. This concept was first described by Mimura et al. using iron-endocytosed rabbit CEC, and later using spheres of CEC precursors.[204-206] Injection of hCEC precursors in a rabbit model confirmed the potential of this approach, as long as a postoperative prone position could be maintained.[207] To improve the number of cells attaching, Patel et al. investigated the incorporation of supermagnetic microspheres into cultured hCEC before injection and applied a magnetic field to direct cells towards the posterior cornea in an *ex vivo* model.[208]

In 2012, the use of ROCK inhibitor as an adjuvant to promote hCEC adhesion to the posterior cornea after cell injection was suggested,[209,210] after the same group reported that the ROCK inhibitor Y-27632 enhanced adhesion and inhibited apoptosis of monkey CEC *in vitro,* and enhanced corneal endothelial wound healing *in vivo* in animal models.[211,212]

In vivo application of cell injection in animal models

Additional work was conducted with monkey and rabbit models, to further explore the concept of cell injection therapy.[44,213,214] In the initial experiments, the recipient's cells were removed by scraping but Okumura et al. later showed, in a rabbit model, that a small descemetorhexis of 4 mm could be performed.[214] Recently, hCEC injection was compared with the hCEC delivered on a tissue-engineered graft of human origin in a rabbit model of bullous keratopathy.[215] Results showed that both approaches were effective in reversing corneal blindness in the rabbit model. Transplanted hCEC retained their hexagonal shape and expressed phenotypical human cell markers, regardless the modality of delivery. In another rabbit model, the injection of a CEC suspension was compared with the injection of CEC mini-sheets, which were aggregates of 4-10 CEC derived from the enzymatic dissociation of confluent cultured rabbit CEC with accutase.[216] CEC mini-sheet injection restored corneal clarity and thickness in the rabbit eye after 7 days of injection, while for single CEC suspension this was observed after 14 days of injection.

A combination of the "modified" dual media approach[47] and cell injection was recently explored by Ong et al. After storage of peeled DM-EC tissue in a growth-factor-depleted medium for 48 hours, non-cultured single hCEC obtained after enzymatic treatment of DM-EC showed a smaller cell morphology and an overall cellular yield compared to single cells derived from digestion of DM-EC stored in a F99-based medium with growth factors. After non-cultured single cells injection in a rabbit bullous keratopathy model, corneal thickness became increasingly thinner and corneas remained clear at 3 weeks after injection.[217] Unlike published methodologies, this approach does not require complex cellular propagation techniques.

Clinical trials

A milestone for endothelial cell injection was the first-in-man clinical trial conducted by the Kinoshita group. The first patient reported showed resolution in the corneal edema, and experienced an improvement in visual acuity from 0.04 to 1.0 Decimal Snellen best corrected visual acuity (BCVA).[218] Results of a group of 11 patients, that were treated for BK (FECD n=7), showed clinical improvement and an increase in ECD after injection of a ROCK inhibitor-supplemented cell suspension.[23] Following the mechanical removal of the aberrant ECM on the patient's DM and/or of the degenerated CEC in an area of 8-mm diameter, a suspension of 10^6 cells was injected into the patient's anterior chamber. After the procedure, patients were placed in a prone position for 3 hours, to allow the sedimentation of the injected hCEC onto the posterior surface of the cornea. At 24 weeks after cell injection, improvements in ECD, corneal thickness decrease, and best corrected visual acuity were observed. Two years after cell injection, the corneal thickness was less than 600 μm in 10 eyes out of 11, while each of the 11 eyes retained corneal transparency. No immune response to hCEC was observed.[23]

Within the limits of the small study group, the procedure was safe and no severe adverse events were reported. However, safety issues still need to be better defined before applying this technique on a large scale.[23,219] The fate of unattached cells and their effect upon entering the trabecular meshwork or the systemic circulation is not yet fully understood.

An alternative approach for cell injection therapy was recently presented by Parikumar et al. with a small case series of three patients.[220] They first placed a nanocomposite gel sheet in the anterior chamber[221] and then injected a suspension of hCEC precursors in the space between the posterior cornea and the gel sheet. The gel sheet was intended to facilitate cell attachment and was removed three days after cell injection. After 6 months, bullae in all three analyzed eyes had resolved and did not re-occur within the 18-month follow-up period. No information on ECD was presented in the study.

REGULATORY COMPLIANCE

Clinical application of cultured hCEC requires compliance with strict regulatory guidelines. These guidelines may not be identical in all countries as they are set by local regulatory authorities,[222-228] but have the common goal to ensure safety and reproducibility. Cell isolation and culture protocols must adhere to GMP requirements because these treatments are considered advanced therapeutic medicinal products (ATMP) according to the European legislation, human cells, tissues and cellular and tissue-based products (HCT/P) as for United States' FDA regulation, and regenerative medical products (RMP) for the Japanese guidelines. A major challenge for GMP compliance is the use of animal-free components to reduce the risk of disease transmission and the batch-to-batch variability.

Collagenases used for cell isolation are produced by different bacteria such as *Clostridium histolyticum*, but the production is related to the fermentation of animal-derived nutrients.[229] GMP-compliant enzymes used in the hCEC isolation process include Liberase TH, an enzymatic blend without animal-derived components and mainly composed by defined ratios of highly purified Collagenase I and Collagenase II and with a high percentage of Thermolysin.[46,49,215] A comparative analysis of Liberase TH with the commonly used research-grade Collagenase I showed no differences in terms of both optimal working concentration for the digestion of the DM-EC complex and overall cell concentration obtained per cornea.[46] Collagenase NB may be another GMP-compliant option, as this enzymatic blend of Collagenase I/Collagenase II with the addition of Clostripain[230] has been used for the isolation of cells from many tissues.[231-235] For DM-EC digestion, the use of TrypLE™ Select has been suggested for GMP-compliant protocols and authors described a similar dissociation rate of confluent hCEC between TrypLE™ Select and another TrypLE™ reagent.[46]

In the vast majority of reported hCEC culture protocols, culture medium is supplemented with animal-derived fetal bovine serum (FBS).[38,40,41,43,45-48,61,137] Use of Equafetal was approved for clinical trials by FDA and pharmaceutical regulatory agencies of UK and Japan. However, while it is derived from animals held in controlled diet and living conditions, it is still a source of animal

components.[46] Other studies reported culture medium supplementation with human-derived serum[236] and human platelet lysate,[237] or even the establishment of hCEC cultures expanded using serum-free culture media,[32,46] though the success rates were variable.

The materials used for coating of cell culture surfaces are another potential source of xeno-contamination. Recombinant proteins such as laminin-511 and laminin-521 are already available on the market,[90] while the natural-derived pericellular matrix from human decidua-derived mesenchymal cells (PCM-DM) has also been shown as a potential xeno-free substrate.[137]

Regulatory authorities require that a quality assessment must be performed on cultured hCEC used for clinical application, to guarantee safety and efficacy for the therapy used on humans. A clinical trial in human patients by Kinoshita et al. included quality assessment features for cultured hCEC used for cell injection, such as: cell viability, visual inspection, cell purity, functional assurance, confirmation of no contamination of the culture medium, and negative testing for bacteria, fungi and viruses.[23] Moreover, several cell surface antigens (CD98, CD166, CD340), as well as cell membrane proteins (SLC4A11), microRNAs and exosomes, have been identified and proposed for quality assessment of cultured hCEC for clinical applications.[238-243]

FUTURE PERSPECTIVES

Corneal transplantation will most likely remain the standard of care for endothelial diseases in the next decade. Motivated by the global shortage of corneal grafts, however, novel therapeutic treatment options have been explored over the last years to either use donor tissue more efficiently or to become independent of donor tissue at all.

For novel approaches based on cultured hCEC, as described in this review, larger clinical trials will be needed to answer questions regarding indication and safety. Potential clinical uptake of this approach will, however, more likely be hampered by costs, as ATMPs are typically very expensive. These regulations also apply to the carrier-based approach, which is currently still in the preclinical stage. Approval for a first-in-man clinical trial for tissue-engineered corneal grafts was recently granted by Health Sciences Authority in Singapore (Clinical Trial Certificate: CTC1800013) for the treatment of FECD and bullous keratopathy.[212]

To further pursue the transplantation of cultured hCEC on carriers, additional research efforts will need to be invested in developing suitable carriers. While natural carriers closely mimic the native DM, availability is limited and subjected to donor-to-donor variability. The focus may therefore be best placed on bioengineered carriers which would be a potentially unlimited source for tissue-engineered grafts, and with the possibility to finely tune the biomechanical properties. Current challenges for bioengineered grafts are ensuring sufficient cell adherence and pumping function of transplanted cells on the carriers.

An interesting future aspect of the cell-based approaches may be the possibility to apply non-HLA matched allogeneic cells.[244-246] The alloimmunogenicity of murine primary CEC (mpCEC) that were injected into the anterior chamber of a mouse CEC transplantation model, created by excising mpCEC from the recipient murine cornea.[247] Results showed that injected mpCEC did not provoke an allogeneic delayed-type hypersensitive (DTH) response. Moreover, mpCEC that adhered to the recipient murine cornea induced allograft tolerance 8 weeks after injection, indicating that mpCEC transplantation may have a degree of protection from allogeneic rejection.

In parallel to developing cell-based approaches, also other approaches will continue in pursuit of becoming more independent from donor tissue. *In vitro* results for in-office approaches to perform laser-based descemetorhexis or selective guttae removal have recently been presented,[248,249] and may be combined with topical medication of ROCK inhibitors.[44,207,218] For gene therapy for FECD different approaches have been investigated.[250,251] Until the above-mentioned approaches can be applied clinically, current tissue-efficient or tissue-sparing techniques such as Quarter-DMEK and Descemet stripping only will remain the main treatment alternatives to standard corneal transplantation.

CONCLUSION

In conclusion, promising new developments targeting endothelial donor tissue shortage are emerging. While the first clinical results for surgical-based approaches and cell injection therapy are available, engineered endothelial cell sheet transplantation is still in the pre-clinical stage. For all approaches, more studies are needed to evaluate whether these techniques have indeed the potential to complement the available corneal transplantation techniques as a treatment option for endothelial diseases.

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CHAPTER 3

Adapted from:

Improving the success rate of human corneal endothelial cell cultures from single donor corneas with stabilization medium

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ABSTRACT

The main objective of this study was to improve the success rate of human corneal endothelial cell (hCEC) cultures from single donor corneas. We could show that the use of stabilization medium prior to cell isolation has a positive effect on the success rate of hCEC cultures from single research-grade donor corneas by allowing growth of otherwise possibly not successful cultures and by improving their proliferative rate. hCEC were obtained from corneo-scleral rims of 7 discarded human research-grade cornea pairs. The Descemet membrane–endothelium (DM–EC) sheets of each pair were assigned to 2 experimental conditions: (1) immediate cell isolation after peeling, and (2) storage of the DM–EC sheet in a growth factor-depleted culture medium (i.e. stabilization medium) for up to 6 days prior to cell isolation. hCEC isolated by enzymatic digestion were then induced to proliferate on pre-coated culture plates. The success rate of primary cultures established from single donor corneas were higher for DM–EC sheets kept in stabilization medium before cell isolation. All cultures (7/7) initiated from stabilized DM–EC sheets were able to proliferate up to the third passage, while only 4 out of 7 cultures initiated from freshly peeled DM–EC sheets reached the third passage. In addition, for the 4 successful paired cultures we observed a faster growth rate if the DM–EC sheet was pre-stabilized prior to cell isolation (13.8 ± 1.8 vs 18.5 ± 1.5 days, $n = 4$, $P < 0.05$). Expression of the phenotypical markers Na^+/K^+-ATPase and ZO-1 could be shown for the stabilized cultures that successfully proliferated up to the third passage.

KEYWORDS: human corneal endothelial cells, stabilization medium, cell culture, cell isolation, cell morphology, cell viability.

INTRODUCTION

Human corneal endothelial cells (hCEC) are crucial for maintaining corneal transparency, since loss of their functionality owing to endothelial diseases or trauma, results in corneal swelling and loss of corneal clarity.[1,2] Because of the limited proliferation capacity of hCEC *in vivo*,[3] replacement of diseased or damaged endothelium by healthy donor cells by means of corneal transplantation, is currently the only effective treatment option to restore patients' vision.[4] Over the last decade, corneal transplantation for treating endothelial disease, has swiftly advanced from full-thickness penetrating keratoplasty, to the more selective endothelial keratoplasty techniques. Descemet membrane endothelial keratoplasty (DMEK) is the most selective of these techniques to date and specifically replaces the recipient's diseased endothelium and Descemet membrane (DM) by a healthy donor DM–endothelium (DM–EC) sheet.[5,6] Although this method has several advantages over traditional penetrating keratoplasty (shortens the recovery time, reduces the risk of inflammation and graft rejection), one of its limitations is the shortage of high quality healthy donor tissue. This has led to considerable interest in the development of new strategies to increase the pool of available donor tissue, such as the introduction of hemi- and quarter-DMEK,[7,8] in which the donor DM–EC is divided in 2 and 4 pieces, respectively, allowing a much more efficient use of donor tissue.

Transplantation of *in vitro* expanded cultured hCEC from healthy donor corneas, would be an alternative approach that could possibly solve donor tissue shortage.[9,10] In most *in vitro* culture protocols, hCEC isolated from several donor corneas are pooled together and induced to proliferate.[11,12] However, results have been variable and often with limited success.[13,14] In addition, this approach might not be suitable for future clinical application, because of lack of donor traceability and increased antigen load that would significantly increase the risk of allograft rejection. Therefore, we aimed to culture hCEC from single donor corneas. However, one of the main challenges here is the establishment of a reproducible protocol for the *in vitro* propagation, since the proliferative capacity of hCEC is influenced by many factors including cell density, and this may be lower than required when isolating hCEC from one single donor cornea. A low hCEC density at initiation of culture may induce endothelial-to-mesenchymal

transition and might have a general negative impact on morphology and proliferation *in vitro*.[15]

In a recent report by Peh et al., a dual media culture approach before passaging cultured hCEC was described, in which a serum-supplemented medium was shown to prevent endothelial-to-mesenchymal transition of hCEC expanded in proliferative medium and to conserve hCEC morphology *in vitro*.[16] Based on this, we hypothesized that preserving the entire DM–EC sheet prior to hCEC isolation in a similar serum-supplemented medium with no added growth factors (stabilization medium) would be beneficial for culturing hCEC from single donor corneas (i.e. without pooling several donor corneas to establish a culture). To minimize the effect of donor variation, we chose a paired donor cornea approach in which hCEC of one cornea of each pair were immediately isolated after peeling the DM–EC sheet, whereas the DM–EC sheet of the contralateral cornea was kept in stabilization medium for 4–6 days prior to hCEC isolation. The success of establishing stable hCEC cultures as well as their growth rates were assessed.

MATERIALS AND METHODS

Materials

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (PBS), TrypLE™ Express (TE), ascorbic acid 2-phosphate, collagenase from Clostridium histolyticum (Type A), paraformaldehyde (PFA), bovine serum albumin (BSA), 4',6-diamidino-2-phenylindole (DAPI), and Triton X-100 were purchased from Sigma-Aldrich Chemistry BV (Zwijndrecht, The Netherlands). Pen/Strep Pre-Mix was purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Fibronectin, collagen, and albumin (FNC) coating mix was purchased from Athena ESTM (Baltimore, MD, USA). Antibodies were obtained from Life Technology Europe BV (Bleiswijk, The Netherlands). Trypan Blue solution 0.04% (Hippocratech, Rotterdam, The Netherlands) was used to assess the vitality of hCEC during the isolation and culture protocol as well as to ensure the visibility of the DM–EC sheet during preparation.

Research-grade human corneoscleral tissues

Seven discarded research-grade human cornea pairs from 2 female and 5 male donors with a mean age of 69 (± 15) years (range 42–80 years, **Table 1**) were included in the study. All donor corneas were obtained from Amnitrans EyeBank Rotterdam and had an intact and viable endothelium, but were unsuitable for transplantation. In all cases, the donors had stated to have no objection against transplant-related research.

Donor tissue protocol

Primary hCEC were isolated from DM–EC sheets using a two-step, peel-and-digest method. The proto-col for harvesting the DM–EC sheets has been described previously.[17,18] Briefly, after decontamination of the globes, corneo-scleral rims were excised within 36 h post-mortem and stored in preservation medium (CorneaMax, Eurobio, Courtaboeuf, France) at 31ºC until further processing. To peel the DM–EC sheet, corneo-scleral rims were placed

endothelial-side-up on a custom made holder with a suction cup. DM–EC was then stained with 0.04% Trypan Blue solution for 10 s to visualize Schwalbe's line. DM–EC including trabecular meshwork was loosened over 360º. By holding the trabecular meshwork with fine forceps and making gentle centripetal movements, the DM–EC sheet was carefully peeled from the posterior stroma. After removing the trabecular meshwork, a 'Descemet-roll' formed spontaneously with the endothelium laying on the outer side. The DM–EC sheets obtained from each pair as described above were processed further by (1) immediate isolation of hCEC from the DM–EC sheet (non-stabilized hCEC), and (2) by storing the entire contralateral DM–EC sheet in stabilization medium (SM, Table 2) (stabilized-hCEC) first for 4–6 days before hCEC isolation.

Donor information	**Indicators**
Gender, n	
Male	5
Female	2
Mean age (±SD), yrs (range)	69 (±15), (42-80)
Mean storage time (±SD), days (range)	16 (±5), (9-23)
Mean ECD (±SD), cells/mm^2 (range)	2200 (±372), (1700-2700)
Cause of death, n	
Cardio/Stroke	2
Other	2
Infectious	1
Respiratory	1
Cancer	1

Table 1: Demographics of donor data.

Isolation and growth of human corneal endothelial cells

For both conditions (non-stabilized and stabilized DM–EC sheets), hCEC were isolated from the DM–EC sheets by exposing them to a 2 mg/ml collagenase A (in DMEM) solution for 3–6 h at 37ºC and 5% CO_2 to dislodge hCEC from DM, which resulted in tightly packed hCEC clusters (**Figure 1**). hCEC clusters were further dissociated into single cells with TrypLE™ for 5 min at 37ºC and the resulting cell suspension was centrifuged at 500 rpm for 5 min at 37ºC. The cell pellet was re-suspended in Proliferation Medium (PM, **Table 2**) and plated onto culture well plates previously coated with FNC coating mix. From each culture, 10 µl of cell suspension was collected to perform an automatic cell counting using a Spark™ 10 M multimode microplate reader (Tecan Trading AG, Männedorf, Switzerland). The cultures were kept in a humidified atmosphere at 37ºC and 5% CO_2. For routine maintenance, every 2–3 days medium was replaced with fresh proliferation medium. When primary cultures of hCEC reached the stationary phase with 80–90% confluence (approximately after 3 weeks) (**Figure 1**), proliferation medium was replaced with stabilization medium for the next 2–4 days before passaging to enhance the morphology of the expanded hCEC.[16,19] Upon passaging, cultured hCEC were treated with 0.05% Trypsin/0.02% EDTA solution (TE) for 15 min at 37ºC and 5% CO_2, the cell pellet was re-suspended in proliferation medium, and cells were sub-cultured at a 1:2 splitting ratio on FNC-coated culture well plates. The morphology of the cultured hCEC at confluence and during expansion was observed with an AxioVert.A1 microscope with AxioCam ERc 5 s stand-alone functionality camera (Zeiss, Oberkochen, Germany).

Immunofluorescence

ZO-1 and Na^+/K^+-ATPase are phenotypical markers for hCEC. To visualize ZO-1 and Na^+/K^+-ATPase, hCEC were cultured either on glass coverslips or directly on FNC-coated well plates and fixed in 4% paraformaldehyde for 15 min at room temperature. Following fixation, hCEC were first washed with PBS and then permeabilized using permeabilization buffer (0.1% Triton X-100 in PBS) and then rinsed with blocking buffer (5% bovine serum albumin (BSA) in PBS) for 1 h to prevent non-specific staining. Blocking buffer was also used for

primary and secondary antibody dilutions. Incubation with primary antibodies (anti-ZO-1/TJP1 at 1:100, and anti-Na^+/K^+-ATPase at 1:100) was done overnight at 4ºC, followed by a secondary antibody incubation (1:200) for 1 h at room temperature. After washing with PBS, the samples were stained with DAPI to visualize the nuclear DNA, and then imaged using an inverted fluorescence microscope connected to a camera (Axiovert, Zeiss).

Statistical analysis

A paired sample student t-test was performed for outcome comparison between stabilized and non-stabilized DM–EC sheets (SPSS for Windows software, version 15.0, SPSS, Inc.). P values of less than 0.05 were considered statistically significant.

Basal medium	Serum	Growth factors and supplements
(PM) DMEM (Shima, N., et al.; 2011)	15%	2 mM L-Glutamine 2 ng/ml bFGF 0.3 mM L-Ascorbic acid 2-phosphate 10,000 U-ml Pen/Strep
(SM) DMEM (Peh, G., et al.; 2015)	15%	10,000 U-ml Pen/Strep

Table 2: Supplemented media used in the culture of human corneal endothelial cells. PM: proliferation medium, SM: stabilization medium.

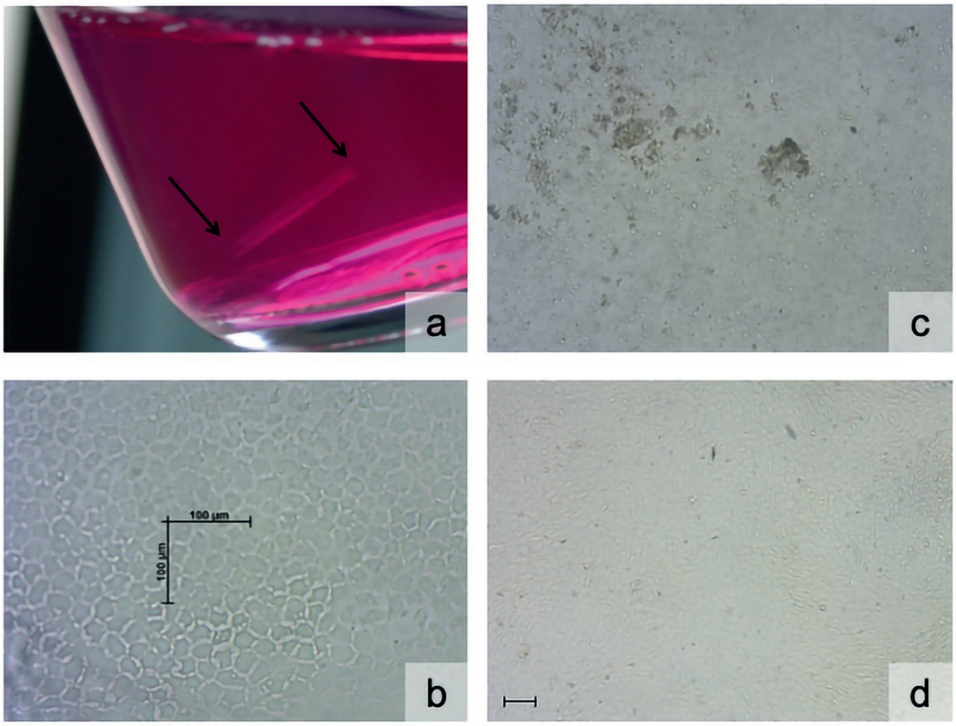

Figure 1: Macroscopic and light microscopic images of the endothelium after DM–EC sheet isolation from a discarded donor corneo-scleral rim. (a) The DM–EC sheet in culture medium. (b) After stripping of the DM–EC sheet, no marked changes in endothelial cells occur throughout the DM–EC sheet. (c) DM–EC sheet after 4 h of digestion in Collagenase A diluted in DMEM. d Confluent hCEC culture at P0 (scale bars = 100 µm).

RESULTS

Endothelial cell density (ECD) determined in the eye bank before DM–EC sheet harvesting was on average 2536 ± 766 cells/mm^2, with no significant difference between the two groups ($P = 0.10$). All other donor-related parameters were identical for both groups due to the paired-donor approach. Cell concentration in both groups (cells/ml) was determined prior to seeding the cells onto the FNC-coated well plates. Average cell concentration was higher for non-stabilized hCEC (7197 ± 5860 cells/ml) than for stabilized hCEC (2435 ± 1656 cells/ml) ($P > 0.05$).

While all cell cultures (7/7) established from stabilized hCEC could be expanded up to the third passage, only 5/7 cultures of the non-stabilized hCEC reached P1 of which 4 could reach P2 (**Table 3; Figures 2, 3**). In these cultures we observed a faster growth rate (time to reach confluence during P0) for stabilized hCEC (13.8 ± 1.8 days) compared to the non-stabilized hCEC (18.5 ± 1.5 days, $P < 0.05$) (**Table 3**) while the characteristic endothelial cobble-stone morphology was maintained (**Figure 2**). Independent of pre-stabilization or not, after the first passage was successful, cell morphology and growth rate were similar between the groups (**Table 3,** donor pairs 2, 4, 5, and 6) (**Figure 2 e, f**). An example of hCEC from one donor pair where the culture was successful independent of prior stabilization is shown in **Figure 3** (**Table 3,** Culture 5)**.**

For the 2/7 non-stabilized hCEC that could not complete P0 (**Table 3,** donor pairs 3 and 7), an abnormal morphology (with elongated fibroblast-like shape) was observed and hCEC were unable to establish confluence. One non-stabilized hCEC culture successfully reached confluence in P0 but not in P1 (**Table 3,** donor pair 1). Here, cells became gradually stretched and also did not obtain confluence.

Expression of the phenotypical markers Na^+/K^+-ATPase and ZO-1 could be shown for the stabilized hCEC that successfully reached P2 (**Figure 4**). Na^+/K^+-ATPase expression had a more diffuse pattern all over the cell surfaces, whereas ZO-1 was mostly expressed on the cell borders.

Passage	Donor Pair 1		Donor pair 2		Donor pair 3		Donor pair 4		Donor pair 5		Donor pair 6		Donor pair 7	
	non-SM	SM	non-SM	SM	non-SM	SM	non-SM	SM	non-SM	SM	non-SM	SM	non-SM	SM
P0	10	13	20	16	-	23	17	14	17	14	20	11	-	14
P1	-	6	6	4	-	6	6	6	6	4	17	4	-	4
P2	-	6	9	7	-	7	4	4	4	4	4	7	-	2

Table 3: Number of days per passage and culture prior to confluence. SM: Stabilization medium prior to cell isolation; Non-SM: no stabilization medium prior to cell isolation: yrs: years.

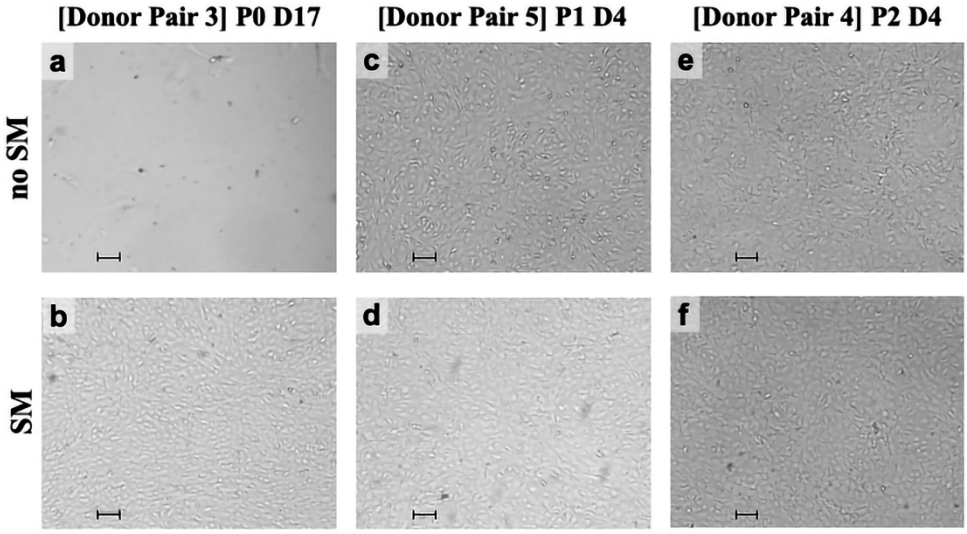

Figure 2: Morphology of cultured hCEC from P0 to P2. Photographs representing the morphology of hCEC isolated from non-stabilized (no SM) and stabilized (SM) DM–EC sheets. Light microscopy images of cultured hCEC are shown for three corneas pairs at initiation of culture (P0), passage 1 (P1), and passage 2 (P2). (a, b) Donor pair 3: P0 at day 17 (D17) at the end of the proliferative phase, before first passaging. (c, d) Donor pair 5: P1 at day 4 (D4), before second passaging. (e, f) Donor pair 4: P2 at day 4 (D4) before third passaging (scale bars = 100 μm)

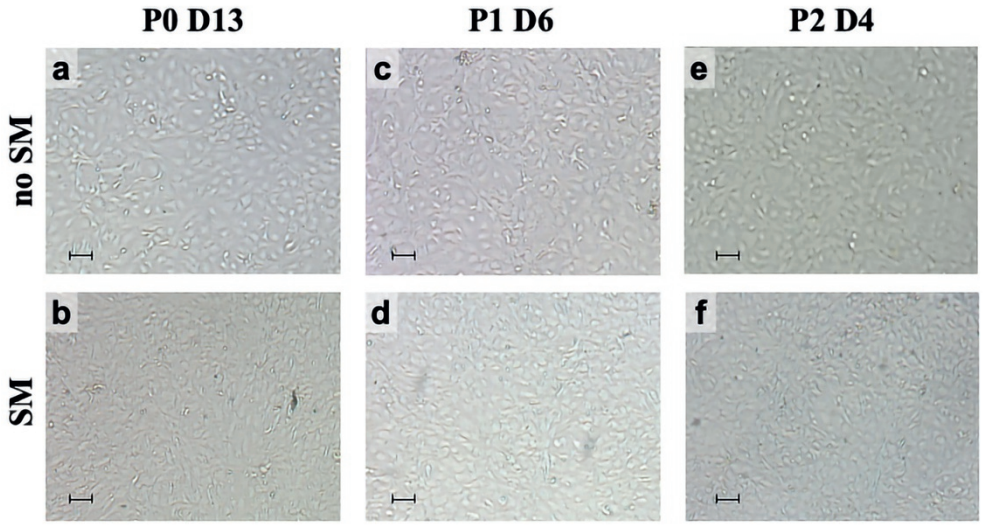

Figure 3: Light microscopy images of successful paired hCEC cultures from P0 to P2. Confluent hCEC cultures isolated from both non stabilized (no SM) and stabilized (SM) DM–EC sheets of donor pair 4. Cell density and morphology were evaluated by light microscopy at P0 (a, b), P1 (c, d), and P2 (e, f) (scale bars = 100 μm).

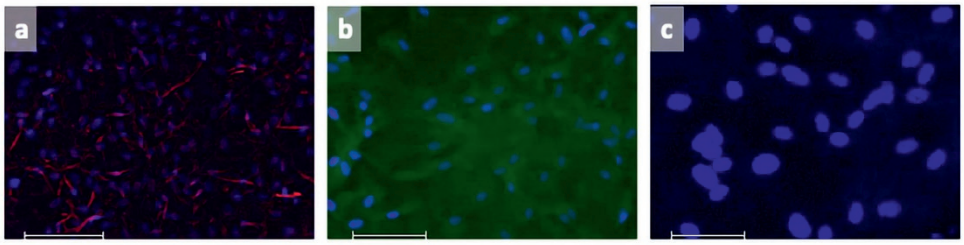

Fig. 4 Characterization and expression of cultured hCEC. Illustrative series of fluorescent images showing the expression of Na^+/K^+-ATPase and ZO-1 of hCEC after passage 2 by immunocytochemistry. (a) Immunostaining of Na^+/K^+-ATPase. (b) Immunostaining of ZO-1. (c) Isotype matched IgG1 negative control. DAPI was used in all experiments for nuclei staining (scale bars = 100 μm).

DISCUSSION

To this day, an impressive amount of protocols for isolation and proliferation of hCEC have been pro-posed, with a main focus on media composition and use of various specific growth factors.[20,21] However, isolation and proliferation of hCEC *in vitro* from human donors remains challenging, especially when cultures have to be established from hCEC which derive from just one donor cornea and the baseline parameters of this research-grade cornea are not optimal. We hypothesized that the success rate of establishing cell cultures of hCEC isolated from single research-grade corneas could be improved by storing freshly peeled DM–EC sheets for 4–6 days in a stabilization medium prior to cell isolation. In this study, we found that 100% of the hCEC cultures initiated from stabilized DM–EC sheets propagated well over two passages whereas cultivated hCEC isolated from the contralateral cornea and expanded immediately after DM–EC sheet harvesting had a success rate of 57%.

We observed that the initial cell concentration before seeding was higher for hCEC isolated from non-stabilized DM–EC sheets than for hCEC isolated from stabilized DM–EC sheets. The most likely explanation for this result is that our stabilization medium affects the presence of viable and non-viable cells. It is known that both the length of organ culture storage time of the corneo-scleral rim,[22-26] and mechanical stress caused by peeling the DM–EC sheet may increase the number of non-viable hCEC.[27,28] This may imply that the initially higher cell concentration before seeding for the non-stabilized hCEC, i.e. isolated immediately after peeling, measured a population of both viable and non-viable cells. The lower cell concentration for the stabilized hCEC may then be explained by a 'loss' of non-viable cells during the stabilization period with mainly viable cells remaining on the DM–EC sheet. Thus, we may find a higher concentration of hCEC after isolating them from non-stabilized DM–EC sheets compared to stabilized DM–EC sheets because of the presence of non-viable cells in the former which are lacking in the latter.

It is known that non-viable cells may negatively impact the cells in their immediate vicinity.[29] Since *in vitro* cell cultures have no mechanism to remove non-viable cells, in the non-stabilized cultures the behavior of viable hCEC may therefore have been negatively influenced by their non-viable neighbors. This

also suggests that by storing the DM–EC sheet for some days in stabilization medium, we were able to isolate and culture a more viable hCEC population. Therefore, it is of the utmost importance to remove the apoptotic cells before seeding the cells, as they produce various factors that may negatively impact their viable neighbors.[30] This may explain the improved growth characteristics of stabilized hCEC compared to non-stabilized hCEC. In three pairs where we could not establish a culture from non-stabilized hCEC, we were able to culture stabilized hCEC from the contralateral cornea over several passages with normal morphology and expression of markers characteristic of human corneal endothelium: Na^+/K^+-ATP and ZO-1.

It is well known that the conditions that may lead to a successful hCEC culture are quite precarious; i.e. an extended storage time before isolation and culture, a high donor age,[2,31,32] and a low yield of hCEC at the start of culture may all negatively affect hCEC propagation.[15] Because of the latter, in most protocols, hCEC are isolated from several research-grade corneas and mixed at initiation of culture,[11,12] which may cause the results to be confounded by donor-to-donor variability. More importantly, this approach is not suitable for eventual clinical application of cultured hCEC because of lack of tissue traceability,[33] and a possible higher risk of allograft rejection because the hCEC originated from different donors. Here, we show that with prior stabilization of DM–EC sheets from research-grade single donor corneas with extended storage time before culture (average 16 days) and a high donor age (average 69 years), we still were able to establish a successful culture in all cases, which was not possible for the non-stabilized contralateral corneas. This result is important for future clinical application of cultured hCEC. Because tissue traceability, and therefore, safety are maintained, the risk of rejection of the cultured hCEC is reduced and even with 'unfavorable' parameters, hCEC may be cultured successfully.
However, it should be pointed out that the study included only a small number of paired corneas (n = 7) of relatively old donors. Therefore it would be interesting to assess the effect of stabilization medium on growing hCEC *in vitro* from a larger number of donors, including donors with an age younger than 50 years since the latter have been shown to propagate better in culture than hCEC from older donors.[2,31,32] Furthermore, viability assays performed on freshly peeled DM–EC and during culture might enable quantification of viable and

non-viable hCEC at any stage during the investigation and might confirm our hypothesis in more detail. Because a Trypan blue staining is not able to discriminate between apoptotic and dead cells,[34] a thorough investigation of various staining methods is required in order to enable the qualitative assessment of the overall cell population prior to cell seeding.

In conclusion, we report a novel straightforward and practical manner to successfully culture hCEC derived from a single donor cornea. This procedure has obvious potential in improving *in vitro* hCEC culture protocols and may aid in the future clinical application of cultured hCEC for corneal endothelial diseases.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST

Dr. Melles is a consultant for DORC International/Dutch Ophthalmic USA and SurgiCube International. Drs. Dapena is a consultant for DORC International/Dutch Ophthalmic USA. The other authors have no conflicting relationship to disclose.

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CHAPTER 4

Effect of GMP-compliant collagenase on the *in vitro* isolation and expansion of human corneal endothelial cells

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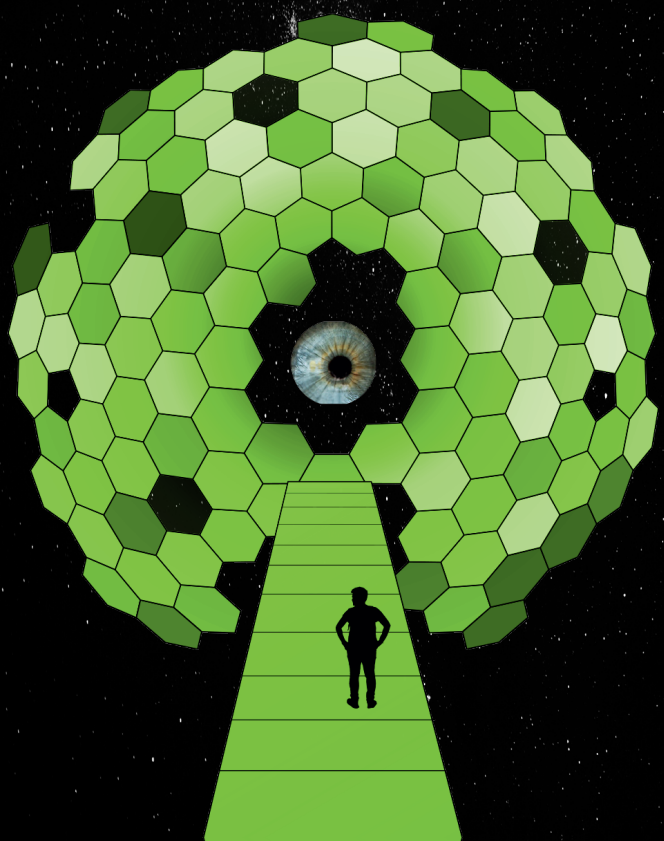

ABSTRACT

Purpose: To compare the effects of two different collagenases on human corneal endothelial cells (hCEC) isolation to potentially improve the culture protocol for research-grade corneas from elderly donors.

Materials and methods: Pre-screening included testing of enzymatic activity of a GMP-compliant collagenase (Nordmark) and a research-grade collagenase A (Sigma Aldrich) by colorimetric assays and cellular viability after collagenase exposure was assessed using human amniotic membranes. Primary hCEC cultures were obtained from 24 research-grade corneas (mean donor age 73 (±8) years) not eligible for transplantation. Descemet membrane- endothelial cell (DM-EC) sheets were exposed to enzymatic collagenase digestion. Time to reach confluence was recorded for each culture and after passaging twice, cells were evaluated by immunohistochemistry.

Results: Pre-screening showed an enzymatic activity of 2.8 U/mg N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA) for the Sigma research-grade collagenase and 0.24 U/mg FALGPA for the Nordmark GMP-grade collagenase, while no significant differences in cell viability following human amniotic membrane digestion (used as a standard calibration method) were observed. Upon hCEC isolation, digestion of DM-EC sheets by the GMP-grade collagenase resulted in the formation of EC sheet fragments rather than hCEC clusters as observed upon DM-EC digestion by the research-grade collagenase. Primary hCEC cultures of cells isolated with the GMP-grade collagenase reached confluence faster than those of cells isolated with the research-grade collagenase (7 (±3) days vs 16 (±8) days, $P<0.05$). Primary hCEC derived from tissue digestion with either collagenase could be successfully passaged once, but after second passage cell cultures of cells isolated with GMP-grade collagenase either failed to reach confluence or displayed polymorphism and exhibited features of cellular stress.

Conclusion: The choice of collagenase can have a profound impact on the hCEC culture protocol for research grade corneas from elderly donors. While the GMP-grade collagenase tested in this study allowed to obtain confluence with high cell density within a week, upon further passage these cells exhibited increasing signs of cellular stress. In given conditions, our results render this

collagenase an unsuitable option for GMP-compliant cell culture protocols for clinical applications.

KEYWORDS: human corneal endothelial cells, cell culture, cell isolation, tissue engineering, GMP-compliant, collagenase.

INTRODUCTION

Cultured human corneal endothelial cells (hCEC) constitute the source for tissue-engineering approaches developed to overcome the global shortage of donor corneas. While cell injection therapy has already been tested in a clinical trial,[1] transplantation of bio-engineered cell-carrier constructs is still in the pre-clinical stage. Both approaches require reproducible cell culture protocols for primary hCEC and compliance with regulatory demands.

Establishing a reproducible protocol for the *in vitro* hCEC propagation is challenging, since the proliferative capacity of hCEC is influenced by many factors, including donor cell density. Low hCEC density at initiation of the culture can promote endothelial-to-mesenchymal transition and might have a general negative impact on morphology and proliferation *in vitro*.[2,3] Therefore, current studies preferably select young donor corneas and those with >2300 cell/mm^2, i.e. donor corneas that would be first line choices for transplantation.[4] Cultivated hCEC from older donors, on the other hand, have lower proliferation potential, and a lower threshold for endothelial-to-mesenchymal transition.[5] Elderly donors, however, constitute the majority of all donors (about 50% of all donors in 2018 in the United States were older than 60 years). Their corneal tissue can be used successfully for corneal transplantation and recently, attempts were presented to address the problem of initiating cell cultures from older donors with low cell density.[6,7] Small modifications to the cell culture protocol improved the success rate for these research-grade corneas and showed that the potential of these corneas has not yet been fully exploited. As cell isolation is one of the critical steps within a robust and reproducible protocol for endothelial cell expansion, in the current study we tested a further modification to the cell culture protocol to compare the effects of two different collagenases on hCEC isolation in order to improve the culture protocol for research-grade corneas from elderly donors. Starting from our previously established hCEC isolation and culture protocol, we compared the proliferation rate of primary cultures derived from hCEC isolated with a research-grade collagenase routinely used until now in our cell culture protocol and a GMP-compliant collagenase not described for being used for hCEC cell isolation until now.

MATERIALS AND METHODS

Materials

Collagenase NB 6 GMP grade from *Clostridium histolyticum* (Cat. No. N0002779, Lot No. 23540115) was obtained from Nordmark Arzneimittel GmbH & Co. KG (Uetersen, Germany) and Collagenase A from *Clostridium histolyticum* (Cat. No. C9722, Lot No. SLBM5274V) from Sigma-Aldrich Chemistry BV (Zwijndrecht, The Netherlands). Dulbecco's modified eagle medium (DMEM), L-ascorbic acid 2-phosphate, paraformaldehyde (PFA), ethylenediaminetetraacetic acid (EDTA), Triton X-100, 4',6-diamidino-2-phenylindole (DAPI), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chemistry BV (Zwijndrecht, The Netherlands). Dulbecco's phosphate-buffered saline (PBS), fetal bovine serum (FBS), TrypLE™ Express, anti-ZO-1/TJP1 primary antibody (#617300), and secondary antibody were obtained from Life Technologies Europe BV (Bleiswijk, The Netherlands). Collagenase Activity Assay Kit was purchased from Abcam (Cambridge, United Kingdom). Pen/Strep Pre-Mix was purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). Fibronectin, collagen, and albumin (FNC) coating mix was purchased from Athena ESTM (Baltimore, MD, USA). Trypan Blue solution 0.04% (Hippocratech, Rotterdam, The Netherlands) was used to estimate hCEC vitality throughout the cell isolation and expansion protocol, and to allow the visibility of the Descemet Membrane-endothelial cell (DM-EC) sheets during preparation.

Pre-screening of collagenases

Pre-screening of the collagenases included comparison of the enzymatic activities of the research-grade collagenase ("Sigma collagenase") and the GMP-compliant collagenase ("Nordmark GMP grade collagenase") by a colorimetric assay (**Table 1**) with Collagenase Activity Assay Kit (Abcam, Cat. No. ab196999, UK). Collagenase stock solutions of 10 mg/mL were prepared with PBS (Sigma Aldrich D8662, USA), containing 0.133 $CaCl_2$ g/L. The collagenase stock solutions were diluted in DMEM (Sigma Aldrich, D1145, USA), containing 0.265 $CaCl_2$ g/L without phenol red resulting in final concentrations of 2

mg/mL or approximately 0.45 PZU/mL, and further diluted from there to suit the range of the assay (1:4.5, 1:9, 1:45). The samples were prepared according to the manufacturer's protocol of the Collagenase Activity Assay Kit. Briefly, samples diluted in assay buffer, positive control, provided by the assay kit, and negative controls (assay buffer) were pipetted into the wells of a 96-well plate (Greiner 96-well microplate, Cat.no. 655101, Austria) all adjusted to a volume of 100 µL/well. The collagenase reaction mix, containing 60 µL collagenase assay buffer and 40 µL bacterial collagenase substrate (N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala (FALGPA)), was added to each well and mixed. Measurement was performed in kinetic mode on a microplate reader (BMG Labtech, Polarstar Omega, Germany). Kinetic measurement was performed measuring every 2 min for 5 – 15 min at 37°C at OD345 nm. Two time points (T1 and T2) within the linear phase of the reaction progress curves were chosen to obtain corresponding OD values at these points (OD1 and OD2). Background control ΔOD value was subtracted from each sample value. Collagenase activity (U/mL) was calculated using the following formula: $((\Delta ODc/\Delta)\ 0.2\ x\ D/0.53\ x\ V)$ = FALGPA [U/mL].

For pre-screening of cellular viability after enzyme exposure, biopsies of human amniotic membrane (8 mm diameter, 6 biopsies) of 3 donors were obtained from Ludwig Boltzmann Institute (Vienna, Austria) and digested under constant shaking with 2 mg/mL Sigma collagenase or 1-4 mg/mL Nordmark GMP grade collagenase for 2 – 3.5 h at 37°C and humidified atmosphere. By this procedure human amniotic mesenchymal stromal cells (hAMSCs) were released from the tissue. Remaining tissue was further digested under constant shaking with 0.05 % trypsin (Sigma, T4174, USA) for 50 min at 37°C and humidified atmosphere, to release human amniotic epithelial cells (hAECs) from the tissue. Both cell populations hAMSCs and hAECS were stained with trypan blue (Merck, Cat. No. 11732, Germany) and viable and non-viable cells were counted in order to determine cellular viability.

Company	Collagenase	Origin	Cat. no.	Lot no.	GMP	PZ activity	FALGPA	FALGPA for 2 mg/mL
Sigma Aldrich	Collagenase A	*C. histolyticum*	C9722	SLBM5274 V	no	-	0.5 – 5.0 units/mg	1-10 U/ml
Nordmark	Collagenase NB 6	*C. histolyticum*	N0002779	23540115	yes	0.225 PZU/mg	-	~ 1.8 U/ml

Table 1: Specifications of Collagenases. Enzymatic activity unit definition: PZ activity (Wünsch) 1 PZ U/mg is estimated to be approximately 3.9 FALGPA U/mg; FALGPA One unit of Collagenase hydrolyzes 1.0 μmole of FALGPA (N-(3-[2-Furyl]acryloyl)-Leu-Gly-Pro-Ala) per minute at 25°C, at pH 7.5 in the presence of calcium ions.

Research-grade human corneoscleral tissues

24 research-grade human corneas not eligible for transplantation and obtained from 20 donors with a mean age of 73 (±8) years (range 58-85 years, **Table 2**) were included in the study. There was no difference in donor age (73 (±7) years vs 73 (±8) years, P=0.47) and in donor endothelial cell density (2258 (±240) cells/mm^2 vs 2117 (±339) cells/mm^2, P=0.06) between the two groups. All donor corneas were obtained from Amnitrans EyeBank Rotterdam and had an intact and viable endothelium. In all cases, donors had stated to have no objection against transplant-related research and the study was conducted according to the tenets of the Declaration of Helsinki and the Barcelona Principles.[8]

Isolation and growth of human corneal endothelial cells

Primary hCEC were isolated from DM-EC sheets using a two-step, peel-and-digest method as previously described.[9,10] After peeling, DM-EC sheets were stored in a growth factor-depleted medium for 4-7 days before hCEC isolation and expansion.[11] Upon tissue digestion, DM-EC sheets were exposed to two different enzymatic treatments: a 2 mg/ml Sigma collagenase (in DMEM) solution, and a 4 mg/ml Nordmark GMP grade collagenase (in DMEM) solution, both for 4 hours. Dislodgement of hCEC from DM was conducted at

37ºC and 5% CO_2 and hCEC clusters were further dissociated into single cells with TrypLE™ Express incubation for 5 minutes at 37ºC. The resulting cell population was plated onto organ-tissue plates previously coated with FNC coating mix and cell density was determined by using 10 µl of cell suspension for an automatic cell count (Spark™ 10M multimode microplate reader, Tecan Trading AG, Männedorf, Switzerland). When reaching 80% to 90% confluence, the culture medium was replaced with stabilization medium for the next 2-4 days before passaging, to enhance the cellular morphology.[12] Primary cultures were then passaged using TrypLE™ Express and sub-cultured at a 1:2 splitting ratio on FNC-coated culture well plates. The morphology of cultured hCEC was assessed throughout the whole culture period with an AxioVert.A1 microscope with AxioCam ERc 5s stand-alone functionality camera (Zeiss, Oberkochen, Germany).

Donor information	Indicators
Number of corneas (donors)	24 (20)
Gender, n	
Male	11
Female	9
Mean age (±SD), yrs (range)	73 (±8), (58-85)
Mean storage time* (±SD), days (range)	14 (±6), (7-25)
Mean ECD (±SD), cells/mm^2 (range)	2188 (±302), (1800-2800)
Cause of death, n	
Respiratory	9
Cardio/Stroke	5
Other	3
Malignant	3

Table 2: Demographics of donor data. *Mean storage time = time between death and culture of first isolated DM-EC tissue; SD = standard deviation; yrs = years.

Immunofluorescence

For hCEC characterization after second passage the expression of the structural marker ZO-1 was analyzed.[13] Briefly, hCEC were fixed with 4% paraformaldehyde in PBS or methanol for 15 minutes at room temperature. Next, samples were permeabilized using 0.1% Triton X-100 and incubated with 3% BSA-based blocking buffer in PBS for 30 minutes. Samples were subsequently incubated with primary antibodies anti-ZO-1 tight junction protein (anti-ZO-1/TJP1, dilution 1:100) for 1 hour, followed by washing steps with PBS and incubation with a secondary antibody (dilution 1:200) in the dark for 45 minutes. Further, the samples were stained with the DNA-specific dye DAPI, and then imaged using an inverted fluorescence microscope.

RESULTS

Colorimetric Analysis of Collagenase Activity and Cellular Viability

Collagenase activity was determined to be 5.6 U/ml FALGPA for Sigma collagenase and 0.48 U/ml FALGPA for Nordmark GMP grade collagenase solutions of the same weight per volume concentration. Accordingly, for 2 mg/mL collagenase solutions, the enzyme activity was determined to be 2.8 U/mg FALGPA for Sigma collagenase and 0.24 U/mg FALGPA for Nordmark GMP grade collagenase (**Figure 1, Table 3**).

To ensure, that the given enzyme concentration had no negative impact during isolation of human cells, human amniotic membrane was degraded for extended times (2-3.5h). Enzymatic tissue degradation of human amniotic membrane with Sigma collagenase or Nordmark GMP grade collagenase with the given activity range had no impact on viability of isolated hAMSCs and hAECs (95.1-100 %).

Digestion of DM-EC sheets and *in vitro* endothelial cell expansion

Based on the colorimetric analysis results which indicated different enzymatic activities for both collagenases (**Table 3**), the Nordmark GMP-grade collagenase was first tested for different concentrations in the range of 1-4 mg/mL and for different digestion times. A concentration of 2 mg/ml for the Nordmark GMP-grade collagenase resulted in very large DM-EC fragments even after digestion times of >4 hours. Raising the collagenase concentration to 4 mg/ml and using digestion times of 4 hours resulted in smaller fragments that were appropriately sized in order to avoid longer trypsin exposure times later in the protocol which might be potentially harmful for the cells.

Enzymatic digestion of the DM-EC sheet by the Sigma collagenase for 4 hours resulted in a total digestion of the DM (**Figure 2A**) and a subsequent flushing step was applied to allow for a homogeneous dislodgement of large hCEC clusters into smaller-sized clusters (**Figure 2B**). In contrast, after enzymatic digestion of the Nordmark GMP grade collagenase exhibited some of the DM to be still attached to the cell sheet after 4 hours of digestion (**Figure 2C**) and

in the following flushing step, the endothelial cell layer fragments were further dislodged from the DM into smaller hCEC clusters (**Figure 2D**).

Cell counting performed on the cell suspensions before seeding showed a higher cell density for the suspensions after digestion by the Nordmark GMP grade collagenase (average seeding density of 18608 ± 10230 cells/ml of cell suspension) compared to the cell density after Sigma collagenase-mediated tissue digestion (average seeding density of 4759 ± 3185 cells/ml of cell suspension) (P=0.04).

During *in vitro* cell expansion, a significantly faster proliferation rate (time to reach cell confluence at P0) was observed in all cultures obtained from DM-EC sheets digested with Nordmark GMP grade collagenase (7 ± 3 days) compared to hCEC cultures established after DM-EC sheet digestion with Sigma collagenase (16 ± 8 days, P=0.03). Cells in both types of culture retained the typical cobblestone shape of endothelial cells (**Figure 3 A,B**). After first passage no variations in proliferation time was observed, regardless the enzyme used from DM-EC sheets digestion (**Figure 3 C,D**). After second passage, however, morphology of isolated cells with the GMP-grade collagenase showed a high degree of variability.

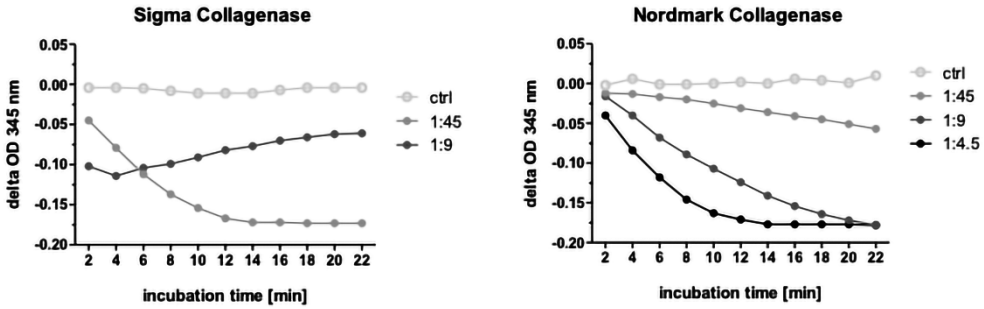

Figure 1: Kinetics of collagenase activities. Kinetics of enzymatic reaction of Sigma Collagenase and Nordmark Collagenase NB 6 GMP grade, measured with Collagenase Activity Assay Kit. OD was measured at 345 nm, and time intervals of measurement were 2 min. Ctrl = negative control. 1:4.5, 1:9, 1:45 = dilutions of the respective collagenase.

Cells appeared larger in size, exhibited signs of cellular stress or were unable to reach confluence at all (**Figure 4 A-C**) as compared to research-grade collagenase-derived cultures (**Figure 4D**). Presence of cellular stress was also reflected by a fragmented expression of ZO-1 at cell borders throughout the layer or showing non-contact inhibited cells (**Figure 5 A,B**).

Collagenase	Collagenase solution (mg/ml)	FALGPA (U/ml)	FALGPA (U/mg)
Research-grade collagenase (Sigma Aldrich)	2	5.60	2.8
GMP-compliant collagenase (Nordmark)	2	0.48	0.24

Table 3. Calculation of Collagenase Activity. Calculations were performed as follows: $((\Delta ODc/\Delta)\ 0.2\ x\ D/0.53\ x\ V)$ = FALGPA [U/mL] (ΔODc = OD2 – OD1 = ΔOD reading from sample at T2 and T1, corrected for background; ΔT = linear phase reaction time T2 – T1 (minutes). 0.2 = reaction volume (mL); D = sample dilution factor; V = sample volume added into the reaction well (mL); 0.53 = millimolar extinction coefficient of FALGPA.

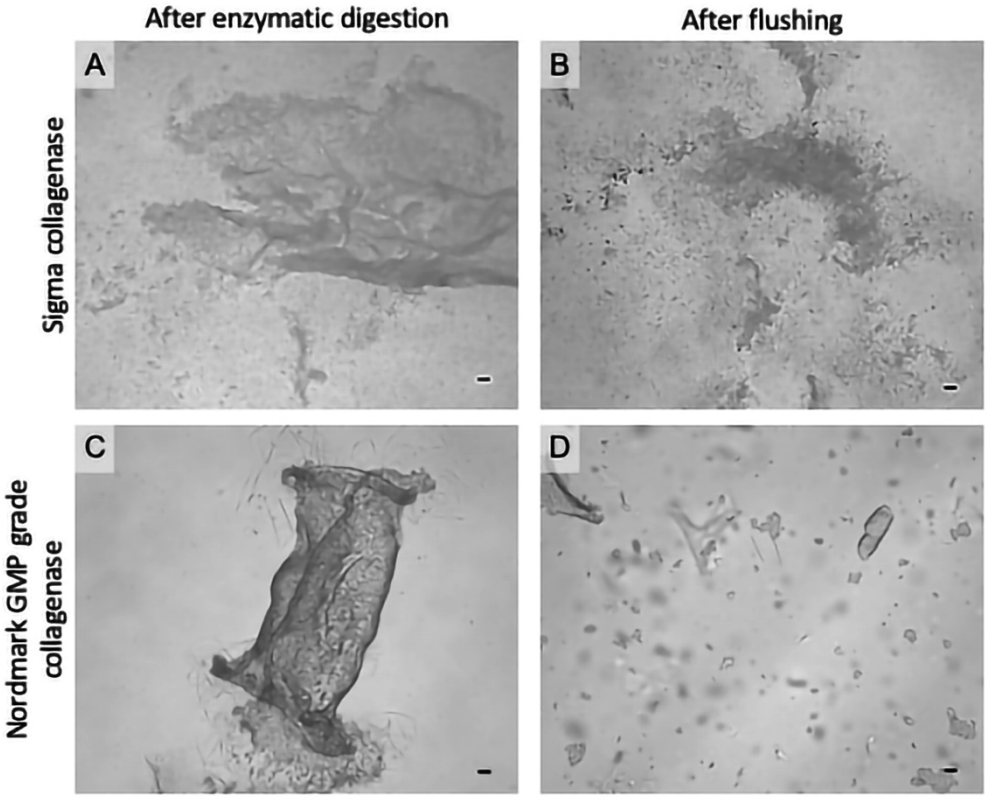

Figure 2: Enzymatic digestion of DM-EC sheets. Series of images illustrating the different outcomes of DM-EC sheet digestion when using Sigma collagenase (A,B) and Nordmark GMP-compliant collagenase (C,D), respectively. Scale bars = 100 μm.

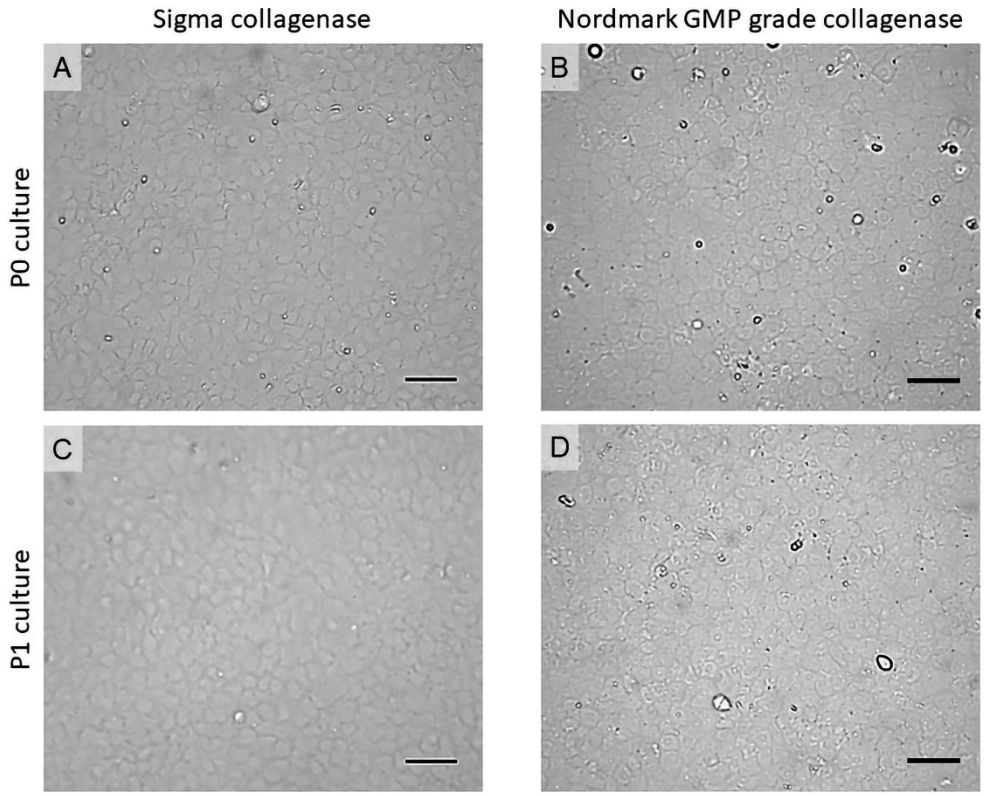

Figure 3: Cultured hCEC isolated from different digestion methods. Light microscopy images representing confluent hCEC cultures from P0 to P1 and isolated from DM-EC sheets following digestion with Sigma collagenase (A,C) and Nordmark GMP-compliant collagenase (B,D), respectively. Scale bars = 100 μm.

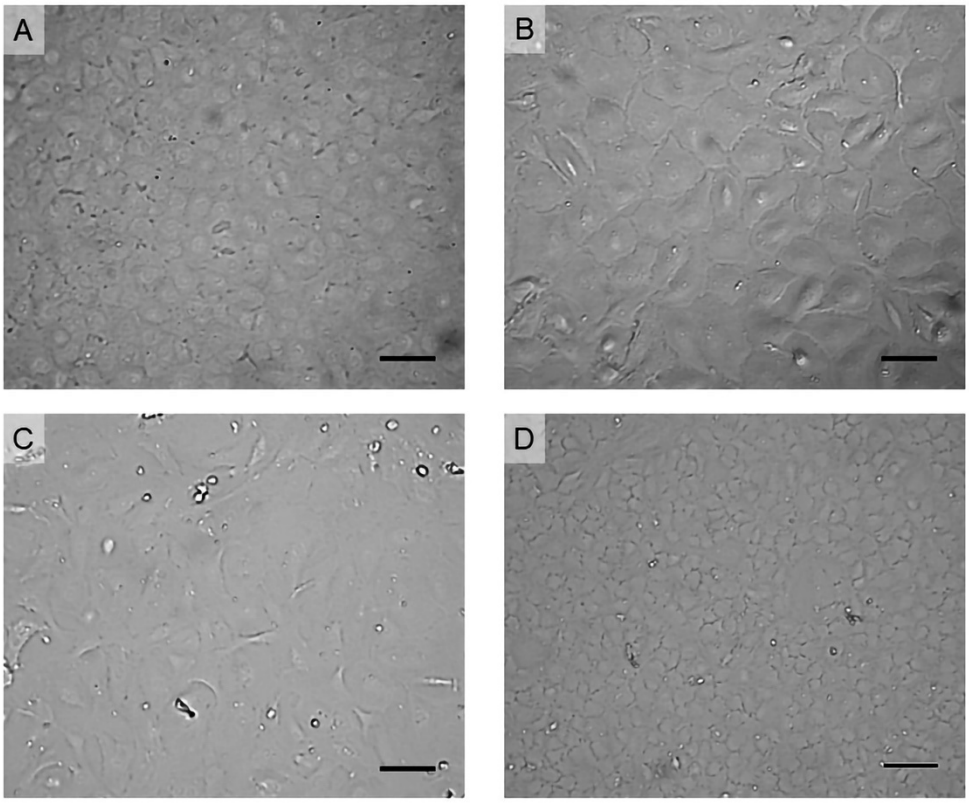

Figure 4: Cultured hCEC at P2. Series of images showing the differences present in hCEC cultures at P2. Cells expanded after isolation with Nordmark GMP-compliant collagenase looked stressed (A), larger in size (B) or were not able to reach cell confluence and good endothelial morphology (C). On the other hand, cultures established following isolation with Sigma collagenase reached a good degree of cell confluence and cells looked smaller (D). Scale bars = 100 μm.

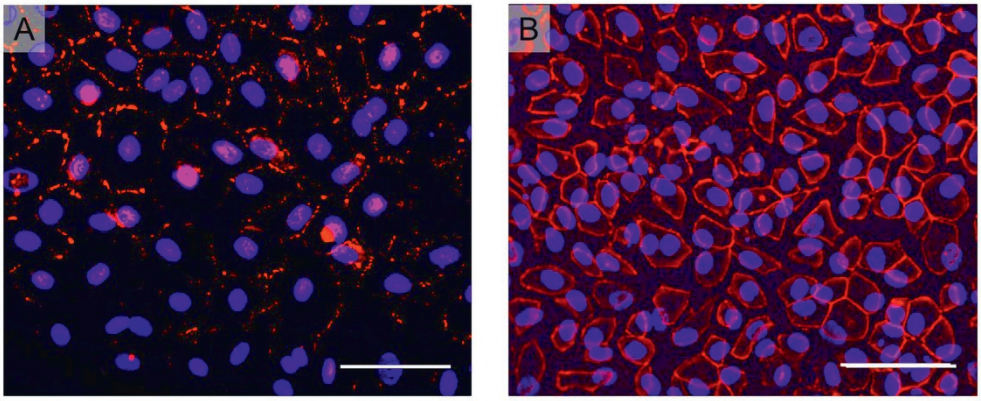

Figure 5: ZO-1 expression on P2 cultures. Immunofluorescence images displaying the expression pattern of the tight junction protein ZO-1 on P2 cultures expanded after tissue digestion with Nordmark GMP-compliant collagenase. Because of the nature of the cells, expression resulted fragmented (A) or highlighted the non-contact inhibition between cells (B). Scale bars = 100 μm.

DISCUSSION

In this study, we analyzed the enzymatic properties of a GMP-grade collagenase and its effect on a previously established *in vitro* hCEC isolation and culture protocol.

The isolation of intact cells from complex tissues still represents a considerable challenge.[14] For corneal endothelial cells, different types of collagenase have been employed for cell isolation, either research-grade or GMP-grade collagenases.[3,12,15,16] Implementation of a GMP-grade collagenase for the cell isolation step is mandatory of compliance with regulatory demands.[17] The GMP-grade collagenase employed in this study had already been successfully applied for isolation of human pancreatic islets,[18] but not for isolation of hCEC. Enzymatic activity of this collagenase proved to be lower than for the research-grade collagenase routinely used in our culture protocol[11] and an increase in its concentration was required for effective DM digestion. Despite the higher concentration, the GMP-grade collagenase appeared to exert a less disruptive effect on the intercellular hCEC connections which in turn resulted in a higher cellular yield. For DM-EC sheets digested with the GMP-grade collagenase, an increase of cellular yield of about 4 times was observed. This is in contrast to results described by Peh et al., who reported no difference in cellular yield when comparing digestion by a GMP-grade collagenase blend (Liberase TH) and a research-grade collagenase (Collagenase Type 1).[19] This might be explained by the superior quality of their research-grade corneas (median donor age of 22 years vs 73 years in this study), resulting in an higher overall cellular yield or by an initial higher yield obtained with Collagenase I compared to Collagenase A.

The higher cell concentration in the seeding cell suspension obtained after the DM-EC sheet digestion with GMP-grade collagenase led on average to shorter times to reach a confluent, uniform cell layer, but with no difference in cell morphology at confluence in cultures derived from the GMP-grade and the research-grade collagenase isolation, respectively. After the first passage, no difference in the time to reach confluence was observed between cultures derived by the two types of collagenases. However, despite the initial high cell density and hexagonal morphology at P0, at second passage cells isolated with the GMP-grade collagenase showed variable outcomes. While some cultures

failed to reach confluence, in other cultures cells exhibited signs of cellular stress. This may indicate that the initial cell isolation process under the conditions applied in this study may induce more cellular stress as compared to the research-grade collagenase, which upon passaging results in a decrease in proliferative capacity. On the other hand, an assay for testing the impact of the applied enzyme concentration on human cells was conducted before starting out the cell isolation experiments and this assay showed no adverse effects of the applied collagenase concentration on amniotic tissue. This would imply that either the assay was not sensitive enough to extrapolate the outcome to human corneal endothelial cells or the cellular stress is induced after the cell isolation step leading to a transient growth arrest in which endogenous telomerase enzymes needed for telomeric overhang from erosion were probably lost in these cells.[20]

It should be pointed out, however, that the higher cellular yield obtained upon tissue digestion with the GMP-grade Nordmark collagenase within this study, had a beneficial effect on starting hCEC cultures derived from elderly donors. Endothelial cells from elderly donors are generally considered as challenging for establishing successful cell cultures. It has been shown earlier that the age of cornea donors represents a critical parameter in the establishment of a reliable and reproducible endothelial cell culture protocol, as these cells have a lower proliferation capacity.[21] However, as the majority of donor corneas derives from elderly donors, these donors constitute a valuable pool of research-grade corneas for transplant-related cell culture studies. In previous studies, modifications to existing cell culture protocols have been suggested to improve the success rate of establishing cell cultures from elderly donors. Parekh et al. reported that forced cell attachment by using hyaluronic acid and supplementing the culture medium with ROCK inhibitor Y-27632 had a beneficial effect on the culture of endothelial cells isolated from old donor corneas.[7] In addition, we described the beneficial use of a modified dual-media approach for establishing confluent hCEC cultures from elderly donors. In that study, research-grade collagenase A was employed for DM-EC sheet digestion.[11] However, a replacement of a research-grade component by a GMP-compliant alternative is essential for fulfilling regulatory demands for potential clinical applications. Since in the current study, tissue digestion by the Nordmark GMP grade collagenase resulted in higher cellular yield than the research-grade collagenase A digestion,

implementing the Nordmark GMP grade collagenase in the cell isolation protocol could have been considered as another step towards establishing a robust cell culture from elderly donor corneas for potential clinical applications. Currently, however, additional studies on the dependency of induced cellular stress on the applied collagenase concentration and how stress induction may be mitigated by additional modifications in the culture protocol may therefore be required before incorporating this tested GMP-grade collagenase into culture protocols.

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CHAPTER 5

Adapted from:

Evaluation of the Suitability of Biocompatible Carriers as Artificial Transplants Using Cultured Porcine Corneal Endothelial Cells

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ABSTRACT

Purpose/Aim: Evaluating the suitability of bioengineered collagen sheets and human anterior lens capsules (HALCs) as carriers for cultivated porcine corneal endothelial cells (pCECs) and *in vitro* assessment of the cell-carrier sheets as tissue-engineered grafts for Descemet membrane endothelial keratoplasty (DMEK).

Materials and Methods: pCECs were isolated, cultured up to P2 and seeded onto LinkCell™ bioengineered matrices of 20 μm (LK20) or 100 μm (LK100) thickness, and on HALC. During expansion, pCEC viability and morphology were assessed by light microscopy. ZO-1 and Na^+/K^+-ATPase expression was investigated by immunohistochemistry. Biomechanical properties of pCEC-carrier constructs were evaluated by simulating DMEK surgery *in vitro* using an artificial anterior chamber (AC) and a human donor cornea without Descemet membrane (DM).

Results: During *in vitro* expansion, cultured pCECs retained their proliferative capacity, as shown by the positive staining for proliferative marker Ki67, and a high cell viability rate (96 ± 5%). pCECs seeded on all carriers formed a monolayer of hexagonal, tightly packed cells that expressed ZO-1 and Na^+/K^+-ATPase. During *in vitro* surgery, pCEC-LK20 and pCEC-LK100 constructs were handled like Descemet stripping endothelial keratoplasty (DSEK) grafts, i.e. folded like a "taco" for insertion because of challenges related to rolling and sticking of the grafts in the injector. pCEC-HALC constructs behaved similar to the DMEK reference model during implantation and unfolding in the artificial AC, showing good adhesion to the bare stroma.

Conclusions: *In vitro* DMEK surgery showed HALC as the most suitable carrier for cultivated pCECs with good intraoperative graft handling. LK20 carrier showed good biocompatibility, but required a DSEK-adapted surgical protocol. Both carriers might be notional candidates for potential future clinical applications.

KEYWORDS: porcine endothelial cells, cell culture, donor material, endothelial cell transplantation, cell carriers.

INTRODUCTION

Corneal endothelial cells (CECs) are essential for the preservation of corneal transparency, since loss of their functionality following endothelial diseases or trauma eventually leads to corneal swelling and edema.[1] Because mature human CECs (hCEC) do not replicate *in vivo*,[1–4] replacement of diseased or damaged endothelium by corneal transplantation is currently the only treatment option.[5] To date, Descemet membrane endothelial keratoplasty (DMEK) specifically replaces the recipient's diseased or damaged endothelium (EC) and Descemet membrane (DM) with the same layers of a healthy donor.[6,7] DMEK provides faster and better visual rehabilitation than other transplantation techniques; however, its application is limited by the shortage of high-quality healthy donor tissue.

One approach to target the shortage of endothelial grafts relies on the *in vitro* expansion of CECs on suitable cell carriers. These tissue-engineered endothelial grafts could become an alternative to the availability of human donor tissue.[8] Previous *in vitro* studies have reported the use of denuded DM and human anterior lens capsules (HALCs), as well as bioengineered matrices consisting of collagen or gelatin, or a combination of biopolymers, as potential carriers for cultured CECs.[9–17] These cell-carrier constructs should preferably be similar to an "original" DMEK-graft, since it has been reported for Descemet stripping (automated) endothelial keratoplasty (DSEK/DSAEK) that thicker grafts may interfere with visual outcome[18] and the biomechanical properties of cell-carrier constructs should allow implantation.

In this study, we evaluated collagen-based bioengineered scaffolds and HALC as carriers for viable cultured porcine corneal endothelial cell (pCEC) sheets. The suitability for transplantation of the pCEC-carrier constructs with the DMEK surgical protocol was tested by simulating DMEK surgery *in vitro*.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (PBS), ascorbic acid 2-phosphate (Asc-2P), paraformaldehyde (PFA), L-Glutamine, trypsin, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), 4′,6-diamidino-2-phenylindole (DAPI), and Triton X-100 were purchased from Sigma-Aldrich Chemistry BV (Zwijndrecht, The Netherlands). Chondroitin sulphate and Pen/Strep Pre-Mix were purchased from Carl Roth GmbH$^+$ Co. KG (Karlsruhe, Germany). Fibronectin, collagen, and albumin (FNC) coating mix was purchased from Athena ESTM (Baltimore, MD, USA). Opti-MEM™ I Reduced Serum Medium (Opti-MEM), bovine pituitary extract (BPE), anti-ZO-1/TJP1 primary antibody, secondary antibodies, and ReadyProbes™ Cell Viability Imaging Kit (Blue/Green) were obtained from Life Technology Europe BV (Bleiswijk, The Netherlands). Anti-Na^+/K^+-ATPase primary antibody and anti-Ki67 primary antibody were obtained from Abcam (Cambridge, United Kingdom). Betadine was obtained from Hippocratech (Rotterdam, The Netherlands). Hypotonic Trypan Blue solution 0.04% (Hippocratech, Rotterdam, The Netherlands) was used to stain the pCEC-carrier constructs during *in vitro* preparation and surgeries. LinkCell™ collagen bio-membranes were provided by LinkoCare Life Sciences AB (Linköping, Sweden).

Porcine corneal endothelial cell isolation

A total of 15 porcine eyes were collected from a local slaughterhouse within 24 h after death. First, porcine globes were decontaminated as described for human eyes[19] and adapted for porcine eyes, including an additional betadine-treatment step before decontamination. Next, the corneo-scleral rim was excised under sterile conditions, placed endothelial side-up in one well of a 12-well plate, and treated with 0.05% Trypsin/ 0.02% EDTA (TE) solution for 20 min at 37°C to cut away the focal adhesion from anchoring the endothelial cells to DM. Trypsin activity was stopped by addition of Opti-MEM containing 8% FBS directly on the corneo-scleral rim. Then, the enzymatically dislodged cells were collected with a sterile pipette tip and centrifuged at 500 rpm for 8 min at 37°C. Based on

the protocol described by Proulx et al.,[20] cells were then re-suspended in fresh Opti-MEM containing 8% FBS, 4 mM L-Glutamine, 200 mg/ml Ca^{2+} chloride, 50 μg/ml BPE, 0.3 mM Asc-2P, 0.08% chrondroitin sulphate, and 100 IU/ml Pen/Strep Pre-Mix (hereafter referred to as "culture medium") and plated in 3 wells of a 6-well plate previously coated with FNC coating mix. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For routine maintenance, every 2–3 days the culture medium was replaced. When primary cultures of pCECs reached 80–90% confluence (approximately after 1 week), they were passaged by treatment with TE solution for 10 min at 37°C and 5% CO_2. Next, the cell suspension was collected, centrifuged at 500 rpm for 8 min at 37°C and the cell pellet was re-suspended in fresh culture medium. The pCECs were sub-cultured at a 1:2 splitting ratio on FNC-coated culture well plates and their morphology at confluence and during expansion was evaluated with an AxioVert.A1 microscope with AxioCam ERc 5s stand-alone functionality camera (Zeiss, Oberkochen, Germany). Use of animal tissue in this study followed the institution's guidelines for animal tissue-based research.

Cell proliferation and viability

Ki67 is a sensitive marker for cell proliferation.[21] To detect Ki67 expression, pCECs at P2 were cultured for 1 week on glass coverslips and fixed in 4% PFA for 15 min at room temperature (RT). Following fixation, samples were washed with PBS containing 0.1% Triton X-100 and then incubated with blocking buffer (3% BSA in PBS) for 30 min at 37°C to prevent non-specific staining. Blocking buffer was also used for primary and secondary antibody dilutions. Samples were incubated with primary antibody anti-Ki67 (dilution 1:100) for 1 h at 37°C and were subsequently washed several times with PBS. Next, samples were incubated with second antibodies (dilution 1:200) for 1 h at 37°C. After washing with PBS, samples were stained with DAPI to visualize the nuclear DNA, and then imaged using an inverted fluorescence micro-scope connected to a camera (Axiovert, Zeiss).

Cell viability was determined on pCECs at P2 cultured on glass coverslips by using the ReadyProbes™ Cell Viability Imaging Kit. Briefly, two drops of each stain (NucBlue® Live reagent and NucGreen® Dead reagent, respectively) were

added per ml of culture media and samples were incubated for 15 min at 37°C in a humidified atmosphere containing 5%CO_2. The cell viability rate was calculated as the average in three areas, each measuring 0.01 mm^2, on digital microphotographs. The percentage of viable cells was determined by manual counting according to the fixed-frame method.

Human anterior lens capsule isolation

HALCs were isolated from human donor eyes at Amnitrans Eyebank Rotterdam. In all cases, the donors had stated to have no objection against transplant-related research. While holding the HALC with the anterior pole up with forceps, an incision into the lens' equatorial area was made using a surgical blade to accomplish a 360° cut. The obtained HALCs were spread epithelial side-up and the lens cortex which was still adherent to the posterior lens cortex was removed using a surgical sponge (Simovision BV, Diemen, The Netherlands) soaked in 70% ethanol. Then, the HALCs were trephined to the desired size (Ø 8.0 mm) from the interior side, and treated with TE solution for 20 min. Next, HALCs were flattened and the loosely attached lens epithelium was removed by sweeping using a sponge soaked in BSS. After rinsing with BSS, because of the elastic properties of the membrane, a "HALC roll" formed spontaneously with the epithelial side on the inside. Each HALC was stored in a glass vial containing 5 ml PBS at 4°C until further use. When ready for cell seeding, the lens capsules were spread with the epithelial side down[16] over FNC-coated glass coverslips in 24-well tissue culture dishes and kept moist in PBS.

Seeding of cultured pCECs on biocompatible carriers

The selected carriers differed regarding their artificial extra cellular matrix (ECM) composition, technological process, and biophysical (strength of cell-substrate adhesion or cell proliferation enhancement) and biomechanical properties (e.g. tensile strength, elasticity). Three different carriers were evaluated in this study: LinkCell™ collagen I bio-membranes of 20 μm and 100 μm thickness (LK20 and LK100, respectively)[22] and HALCs. Diameter of the LK20 and LK100 for the experiments was 9.5 mm.

During the storage time, the carriers were kept hydrated in PBS in order to preserve their biomechanical properties. Primary pCECs, after reaching 80–90% of confluence, were passaged twice (up to P2) before they were seeded onto each the three carriers (LK20, LK100, and HALC). All pCEC-carrier constructs were cultured up to 1 week before they were used for the *in vitro* surgeries.

Immunofluorescence

ZO-1 and Na^+/K^+-ATPase are phenotypical markers for CEC.[23,24] To visualize ZO-1 and Na^+/K^+-ATPase, pCECs at P2 were cultured for 1 week either on glass coverslips or directly on the biocompatible carriers and fixed in 4% PFA for 15 min at RT. Following fixation, samples were washed with PBS containing 0.1% Triton X-100 and then incubated with blocking buffer (1% BSA in PBS) for 1 h at 37°C to prevent non-specific staining. Blocking buffer was also used for primary and secondary antibody dilutions. Incubation with primary antibodies anti-ZO-1 tight junction protein (anti-ZO-1/TJP1, dilution 1:100) and anti-sodium/potassium-ATPase (anti-Na^+/K^+-ATPase, dilution 1:100) was per-formed for 1 h at 37°C and was followed by several PBS washing steps. Next, samples were incubated with secondary antibodies (dilution 1:200) for 1 h at 37°C. After washing with PBS, the samples were stained with DAPI to visualize the nuclear DNA, and then imaged using an inverted fluorescence microscope connected to a camera (Axiovert, Zeiss).

***In vitro* surgeries**

In order to assess suitability for transplantation, surgeries with the pCEC-carrier constructs were simulated *in vitro* by using human anterior remnants (donor corneo-scleral rim without DM and endothelium; donors had stated to have no objection against transplant-related research) mounted onto an artificial anterior chamber (AC; DORC International, Zuidland, The Netherlands).[25] The ability of the pCEC-carrier constructs to form a roll in BSS, their affinity to be stained with Trypan Blue solution 0.04%, their insertion behaviour into the artificial AC, the unfolding behaviour, pCEC-carrier transparency, and ability to adhere to the posterior stroma of the anterior remnant were rated on a 5-point scale (i.e. 0–5),

with higher score indicating greater similarity to *in vivo* DMEK/DSEK behaviour. *In vitro* surgeries with DMEK-grafts were used as a positive control and served as a reference for the scoring on the 5-point scale. All surgeries were performed by two DMEK surgeons (ID, IL).

For the *in vitro* surgery, main steps were performed as for standard DMEK surgery.[26] Three side ports were made at 2, 7, and 10 o'clock limbus, and the artificial AC was filled with PBS. Next, a 3-mm main incision was made at 12 o'clock to insert the pCEC-carrier constructs into the artificial AC. Each of the pCEC-carrier constructs was first stained for visualization with 0.04% Trypan Blue, implanted in the artificial anterior chamber and after unfolding and repositioning the pCEC-carrier constructs against the posterior stroma, the AC was filled 100% with an air bubble for 1 h to support the graft adherence to the stromal surface. Afterwards, air was partly removed through the paracentesis and attachment of the pCEC-carrier constructs to the stroma was evaluated by anterior-segment optical coherence tomography (AS-OCT; Heidelberg Engineering GmbH, Heidelberg, Germany).

RESULTS

Evaluation of cell viability and proliferation during *in vitro* expansion

The pCECs of a native porcine cornea formed a uniform monolayer of tightly packed, hexagonal cells (**Figure 1A**) with expression of the phenotypical markers ZO-1 and Na^+/K^+-ATPase (**Figure 1 B,C**). During *in vitro* cell expansion, the pCECs also displayed a uniform layer of tightly packed cells and showed expression of ZO-1 and Na^+/K^+-ATPase (**Figure 1 D–F**). Positive staining of Ki67 revealed that cultured pCECs retained the proliferative capacity (**Figure 2A**), while a low detection of nuclei of cells with compromised plasma membrane integrity (in green) indicated a high cell viability of the pCEC cultures ($96 \pm 4\%$) (**Figure 2B**) which is also reflected by the low degree of Trypan Blue staining (**Figure 1D**).

pCEC morphology after seeding on different carriers

The pCECs were able to retain endothelial morphology and to form a monolayer composed by closely packed cells when seeded onto LK20, LK100, and HALC (**Figure 3**). In addition, pCECs cultured on LK20, LK100, and HALC showed expression of the phenotypical markers ZO-1 (**Figure 4 A-C**) and Na^+/K^+-ATPase (**Figure 4 D-F**). Na^+/K^+ -ATPase expression had a more diffuse pattern all over the cell surfaces, whereas ZO-1 was mostly expressed on the cell borders.

In vitro surgeries with pCEC-carrier constructs

DMEK-grafts were used as a positive control for *in vitro* surgeries (**Figure 5**) and served as a reference for the scoring (**Table 1**). The pCEC-HALC constructs behaved very similar to a DMEK-graft (**Table 1, Figure 5**) and were implanted using a modified DMEK technique. These constructs naturally rolled on themselves into a double roll, but with the endothelium on the internal surface, unlike a DMEK-graft where the endothelium is on the external surface. Staining of the pCEC-HALC constructs with 0.04% Trypan Blue for visualization compared well to a DMEK-graft (**Table 1, Figure 5A**). The constructs could then be placed in a DMEK glass injector, with its correct orientation, i.e. curls

of the double roll facing downwards, checked under a surgical microscope. The pCEC-HALC carrier was implanted in a DMEK-like normal fashion; combined manoeuvres with air, fluid, and corneal indentation could be used in order to successfully unfold the tissue and position it against the posterior surface of the corneal stroma. Although special caution was needed in order to position the endothelium in its correct orientation due to its "inverse endothelium inside" location when compared with the regular DMEK graft, this was successfully achieved in all cases. One hour post-*in vitro* surgery, after partial air bubble removal, all constructs showed at least partial attachment resulting in an average score of 3.5 (on a scale of 0–5 with 5 resembling a DMEK).

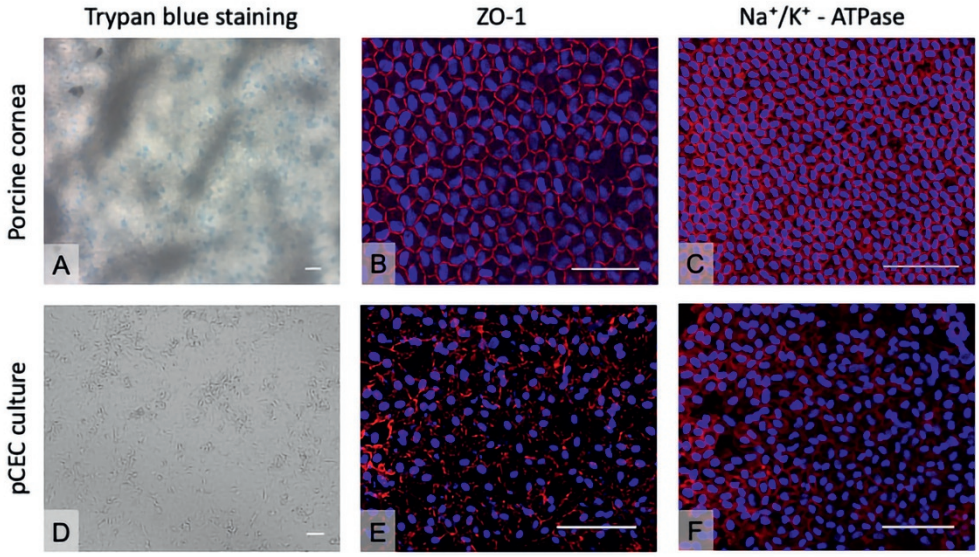

Figure 1: Evaluation of cell morphology on porcine corneas and of pCEC cultures. (A) Porcine cornea stained with hypotonic Trypan Blue solution 0.04%. (B) Expression of ZO-1 detected via immunofluorescence on porcine corneal endothelium. (C) Expression of Na^+/K^+-ATPase detected via immunofluorescence on porcine corneal endothelium. (D) pCECs cultured at P2 upon FNC-coated glass coverslips and stained with hypotonic Trypan Blue solution 0.04%. (E) Expression of ZO-1 detected via immunofluorescence on pCECs cultured at P2 upon FNC-coated glass coverslips. (F) Expression of Na^+/K^+-ATPase detected via immunofluorescence on pCECs cultured at P2 upon FNC-coated glass coverslips. Scale bars: 100 μm.

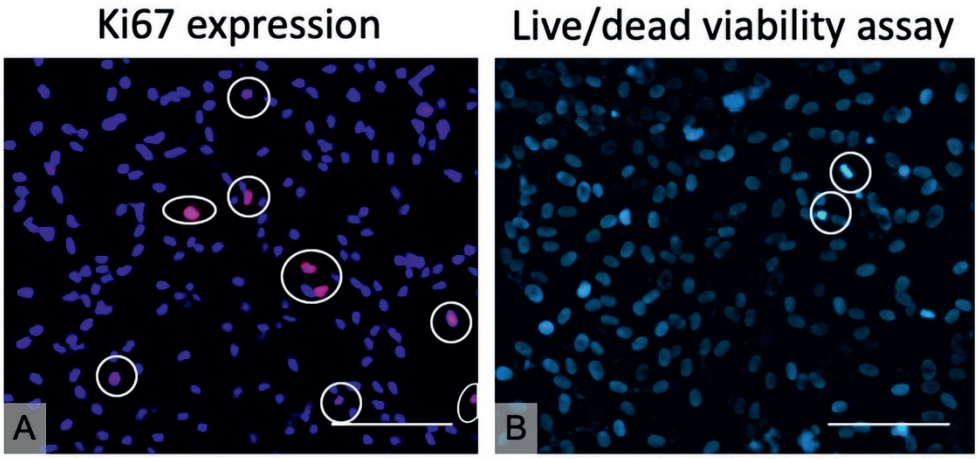

Figure 2: pCEC proliferation and viability at P2. (A) Evaluation of cell proliferation by expression of Ki67 (red, in white circles). Nuclei are stained by DAPI in blue. (B) Live-dead assay to determine cell viability (Blue: Live; Green, in circles: Dead). Scale bars: 100 μm.

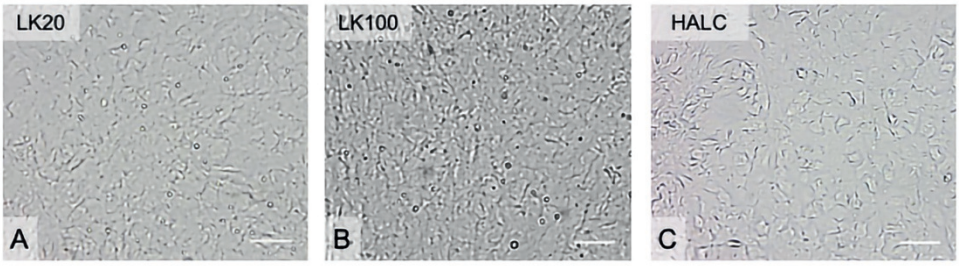

Figure 3: pCEC morphology at P2 on different biocompatible carriers. Series of illustrative figures showing successful pCEC cultures at P2 on chosen biocompatible membranes. (A) LK20, (B) LK100, (C) HALC. Scale bars: 100 μm.

The pCEC-LK20 constructs showed a Trypan Blue staining behaviour similar to the pCEC-HALC constructs and DMEK-grafts (**Table 1, Figure 5E**). These constructs had a tendency to form a large roll in BSS and could be loaded into the injector, but the "sticky" nature of the carrier prevented its easy insertion into the AC compared to a human DMEK-roll. Therefore, the pCEC-LK20 construct was folded over and inserted like a DSEK graft, a procedure in which the endothelial graft includes stromal tissue. Due to the increased thickness and

rigidity, the DSEK-graft does not roll but is folded like a "taco" for insertion (**Figure 5F**). Implantation in the AC and unfolding were more technically demanding compared to a DMEK-graft or the pCEC-HALC construct, as the pCEC-LK20 construct tended to be sticky, fragile, and hard to centralize due to its biomechanical properties (**Figure 5 F–G, Table 1**). Adherence to the bare stroma was also not optimal (**Figure 5H**), resulting in score of 2. Despite Trypan Blue staining (**Figure 5I**), pCEC-LK100 construct did not show any of the essential characteristics of a native DM (**Table 1**). Due to the thickness (100 µm) and increased rigidity of the carrier compared to normal DM, the construct was incapable to roll in BSS, and required a DSEK-like technique for implantation and unfolding (**Figure 5 J–K**) with insufficient adherence (**Figure 5L, Table 1**).

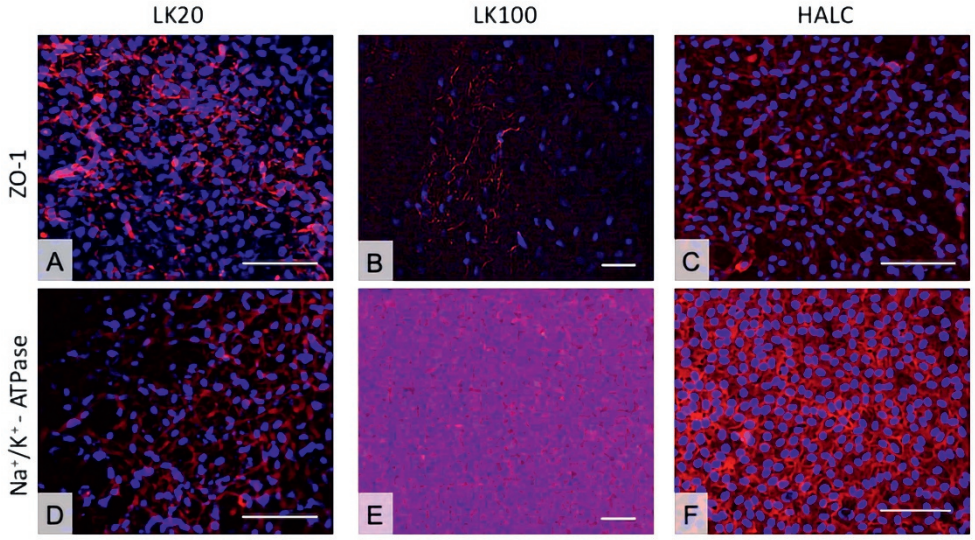

Figure 4: pCEC morphology check at P2 through immunofluorescence on different carriers. (A, B, C) ZO-1 expression detected in pCECs cultured on LK20, LK100, and HALC, respectively. (D, E, F) Na^+/K^+ expression detected in pCECs cultured on LK20, LK100, and HALC, respectively. Scale bars: 100 µm.

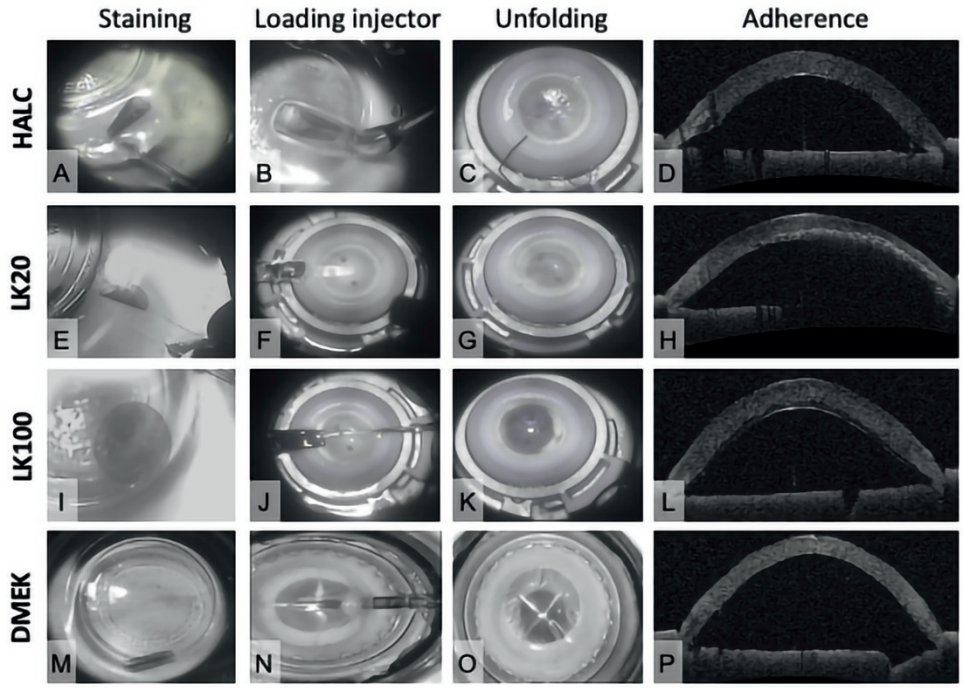

Figure 5: *In vitro* surgeries with pCEC-carrier constructs. The upper three rows show each of the three tested pCEC-carriers constructs after staining with hypotonic Trypan Blue solution 0.04% (A,E,I), insertion into the artificial chamber (B,F,J), unfolding (C,G,K), and adherence to the bare stroma visualized by anterior segment OCT measurement (D,H,L). The lower row (M–P) shows the corresponding images of a DMEK-graft that served as a positive control and reference point for the scoring.

Carrier type	DMEK-graft (positive control)	HALC	LK20	LK100
Rolling in BSS	5	4	1	0
Staining	5	3.5	3.5	5
Anterior chamber	5	4	2.5	1
Implantation		(similar to DMEK, easy orientation)	(lot of manipulation, sticky material)	(rigid material)
Protocol followed	DMEK	DMEK	DSEK	DSEK
Unfolding	5	4 (similar to DMEK, easy to centralize)	2.5 (fragile material, hard to centralize)	0
Transparency	5	4	3.5	0.5
Adherence	5	3.5	2	1

Table 1: *In vitro* surgeries with pCEC-carrier constructs: scoring scheme. Each parameter under evaluation was rated on a 5-point scale (i.e. 0–5) by two DMEK surgeons, with higher score indicating greater similarity to *in vivo* DMEK behaviour. A DMEK-graft was tested as a positive control and served as a reference point for the scoring. Values are reported as means.

DISCUSSION

In this study, we demonstrated that pCECs can be successfully cultured onto biocompatible, bioengineered collagen-based sheets, and onto HALC. Light microscopy and fluorescence microscopy analysis showed successful pCEC cultures up to P2 which retained their characteristic cell morphology, and distinctive expression of endothelial markers such as ZO-1 and Na^+/K^+-ATPase. However, upon *in vitro* transplantation, only the pCEC-HALC constructs behaved similar to DMEK-grafts in terms of staining, rolling in BSS, insertion in the anterior chamber, and attachment to the bare posterior stroma of a human donor corneo-scleral rim (**Figure 5**).

Endothelial keratoplasty is now the preferred treatment for corneal endothelial disease, but scarcity of human donor tissue led to alternative approaches such as *in vitro* expansion of CEC on suitable cell carriers and injection of cultured human CEC.[9,27–29] In previous studies, it was shown that cultivated monkey CEC cultured on a collagen type I carriers for 4 weeks produced a confluent monolayer expressing ZO-1 and Na^+/K^+-ATPase, and at 6 months after transplantation into monkeys, the cornea was still clear with a normal cell density.[30] In addition, hCEC have been shown to grow into a confluent layer on collagen type I-coated culture plates,[31] and cultured hCEC on collagen sheets composed of cross-linked collagen type I trans-planted in rabbits maintained 76–95% of pump function of human donor corneas.[11] Plastic compressed collagen type I, termed Real Architecture for 3D Tissues (RAFT), has been shown to support the growth of hCEC into a confluent mono-layer expressing ZO-1 and Na^+/K^+-ATPase.[32] However, substrates like RAFT have been reported to give rise to inflammation in experimental animal models. In addition, cultivation of CEC on biological carriers such as DM, HALC, and amniotic membrane has been reported.[16,17,28,33]

Ideally, the carrier should mimic DM in its biological and biomechanical characteristics, since this may create a microenvironment required for cellular activity and mechanical support during transplantation using the DMEK technique.[28] To that end, we selected carriers that differed regarding their artificial extra-cellular matrix (ECM) composition, technological process, and

biophysical (strength of cell-substrate adhesion or cell proliferation enhancement) and biomechanical properties (e.g. tensile strength, elasticity).

Our current study showed that pCECs cultured on the LinkCell™ collagen I-based sheets were able to retain endothelial morphology and to form a monolayer composed by closely packed cells with expression of ZO-1 and Na^+/K^+-ATPase. However, the behaviour of the pCEC-LK20 and pCEC-LK100 constructs during the *in vitro* DMEK surgery did not resemble that of a DMEK-graft since they had to be inserted in an artificial anterior chamber like a DSEK-graft (i.e. folded like a "taco") instead of rolled-up like a DMEK-graft.

Our study suggests that HALC may be one approach for use as a biocompatible carrier for CEC, though the need for human-derived tissues is not eliminated through its use and further *in vivo* studies need to be performed. HALC has several benefits as it resembles DM in terms of composition; the major component of HALC is the basement membrane protein collagen IV and other matrix component include collagen types I and III, laminin, fibronectin, and heparin sulphate proteoglycans.[34] The use of HALC as a scaffold for the cultivation of different ocular cells,[35–37] including hCEC,[17,32] has already been reported. It was shown that hCEC seeded on de-epithelialized HALC grew to confluency and strongly expressed ZO-1 and Na^+/K^+-ATPase. We confirm these results with our pCEC-HALC constructs and showed that they can be used successfully in *in vitro* surgeries.

In conclusion, LK20 carrier showed good biocompatibility, but required a DSEK-adapted surgical protocol, while *in vitro* DMEK surgery showed HALC as the most suitable carrier for cultivated pCECs.

DISCLOSURE STATEMENT

Dr. Melles is a consultant for DORC International/Dutch Ophthalmic USA and SurgiCube International. Dr. Dapena is a consultant for DORC International/Dutch Ophthalmic USA.

Dr. Rafat serves on the Board of Directors of the company LinkoCare Life Sciences AB, which is a spin-off firm developing products related to the research being reported, and holds relevant patents. Dr. Rafat's terms of arrangements have been reviewed and approved by Linköping University in accordance with its policy on objectivity in research. The other authors have no conflicting relationship to disclose.

ETHICAL STATEMENT

The study was carried out following the tenets of the declaration of Helsinki.

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CHAPTER 6

Adapted from:

In Vitro Evaluation and Transplantation of Human Corneal Endothelial Cells Cultured on Biocompatible Carriers

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ABSTRACT

Corneal transplantation is among the most effective treatment options for dysfunctional corneal endothelial cells (CEC). In this study, we test *in vitro* the surgical potential of cultivated human corneal endothelial cells (hCEC) on human anterior lens capsule (HALC), LinkCell™ bioengineered collagen sheets of 20-mm thickness (LK20), and denuded Descemet membrane (dDM) as tissue-engineered grafts for Descemet membrane (DM) endothelial keratoplasty (DMEK) to bypass the problem of donor tissue availability. Primary hCEC cultured on all carriers formed a monolayer of tightly packed cells with a high cell viability rate (96% ± 4%). hCEC on HALC and LK20 showed unremarkable expression of zonula occludens-1 (ZO-1) and Na^+/K^+-adenosine triphosphatase (ATPase), while Na^+/K^+-ATPase expression of cells seeded on dDM was mainly cytoplasmic. All hCEC–carrier constructs were evaluated by simulating DMEK surgery *in vitro* using a human donor cornea without DM mounted on an artificial anterior chamber (AC) and a regular DMEK-graft used as a surgical reference model. During *in vitro* surgery, hCEC–HALC constructs behaved most similarly to a DMEK-graft during implantation and unfolding, showing good adhesion to the bare stroma. On the other hand, hCEC–LK20 and hCEC–dDM constructs required some additional handling because of challenges related to the surgical procedure, although they were both successfully unfolded and implanted in the artificial AC. The hCEC–dDM constructs showed similar graft adherence as hCEC–HALC constructs, while adherence of hCEC–LK20 constructs was less effective. After the *in vitro* surgery, the estimated area populated by viable cells on the hCEC–HALC and hCEC–LK20 constructs was ~ 83% and ~ 67%, respectively. Overall, hCEC–HALC constructs behaved most similarly to a DMEK-graft during *in vitro* DMEK surgery, while graft adhesion and surgical handling, respectively, are parameters still requiring optimization for hCEC–LK20 and hCEC–dDM constructs.

KEYWORDS: cell culture, donor material, endothelial cell transplantation, cell carrier, corneal transplantation, DMEK.

INTRODUCTION

Human corneal endothelial cells (hCEC) are vital for maintaining corneal transparency[1]. Loss in hCEC density and functionality due to injury or dystrophic degeneration may lead to corneal edema, haze, and, ultimately if left untreated, to corneal blindness[2,3]. Since hCEC are not thought to replicate *in vivo*[4,5], corneal transplantation is currently the only effective method to restore a healthy endothelial barrier[6]. Over the past two decades, corneal transplantation for endothelial disease has evolved from full-thickness penetrating keratoplasty to thinner, more selective surgeries. The thinnest of all is Descemet membrane (DM) endothelial keratoplasty (DMEK), where the damaged corneal endothelial cells (CEC) and the DM of the recipient are removed and replaced with those of a healthy donor[7,8]. This results in faster visual rehabilitation and better visual acuity. Despite the successful outcomes a major limitation for DMEK, and corneal transplantation in general, is the worldwide shortage of suitable donor tissue[9].

The goal of tissue engineering in corneal grafting is to bypass the problem of donor tissue availability. Endothelial cells can be cultured *in vitro* and injected as a cell suspension but we expect that by attaching them to a scaffold structure, higher cell densities can be achieved, and implantation can be done in a controlled and reproducible manner, with fewer cells being lost to the aqueous flow through the trabecular meshwork[10–12]. Potential cell carrier scaffolds may be described as "natural," that is, derived from the body, like human anterior lens capsules (HALC) or denuded Descemet membrane (dDM), or as bioengineered structures made from collagen, gelatin, or a combination of biopolymers[13–20]. From a translational point of view, a key requirement is that the engineered cell–carrier constructs can be surgically handled and implanted without excessive tissue manipulation, preferably in a reproducible, standardized manner, like a conventional DMEK.

The aim of this study was to assess the suitability of hCEC–carrier constructs for transplantation *in vitro*, following the DMEK surgical protocol as a model. For this purpose, we expanded isolated hCEC on three different carriers: HALC, collagen-based bioengineered scaffolds, and DM deprived of their endothelial cells (dDM).

MATERIALS AND METHODS

Materials

Collagenase from *Clostridium histolyticum* (type A), Dulbecco's phosphate-buffered saline (PBS), fetal bovine serum (FBS), L-glutamine, ascorbic acid 2-phosphate (Asc-2P), fibroblast growth factor (FGF), TrypLE™ Express (TE), trypsin, Dulbecco's Modified Eagle's medium (DMEM), paraformaldehyde (PFA), ethylenediaminetetraacetic acid (EDTA), Triton X-100, 40,6-diamidino-2-phenylindole (DAPI), Calcein-AM, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chemistry BV (Zwijndrecht, the Netherlands). Fibronectin, collagen, and albumin (FNC) coating mix was purchased from Athena ESTM (Baltimore, MD, USA). Pen/Strep Pre-Mix was purchased from Carl Roth GmbH þ Co. KG (Karlsruhe, Germany). Anti-Na^+/K^+-ATPase primary antibody and anti-Ki67 primary antibody were obtained from Abcam (Cambridge, UK). ReadyProbes™ Cell Viability Imaging Kit (NucBlue® Live reagent and NucGreen® Dead reagent), anti-ZO-1/tight junction protein (TJP)1 primary antibody, and secondary antibodies were purchased from ThermoFisher Scientific Europe BV (Bleiswijk, the Netherlands). Hypotonic Trypan Blue solution 0.04% (Hippocratech, Rotterdam, the Netherlands) was used to assess the vitality of hCEC during the isolation and culture protocol, to ensure the visibility of the DM-EC sheet during preparation and to stain the hCEC–carrier con-structs during *in vitro* preparation and surgeries.

Research-Grade Human Corneoscleral Tissues

Twelve research-grade human corneas ineligible for transplantation but with intact and viable endothelium, obtained from Amnitrans EyeBank Rotterdam and with a mean age of 67 (±12) yr (range 54–83 yr, **Table 1**), were included in the study. There were no statistically significant differences among the average donor ages of the corneas used to isolate cells to be seeded on HALC, on LinkCell™ bioengineered collagen sheets of 20-mm thickness (LK20), and on dDM, respectively (70 (±9) vs 67 (±13) vs 74 (±13) yr, all $P > 0.05$). Other baseline donor characteristics are listed in Table 1. All donors of tissue used in this study had stated to have no objection against transplant-related research and

the study adhered to the tenets of the Declaration of Helsinki and the Barcelona Principles[21].

Donor information	Indicators
Number of corneas (donors)	9 (5)
Gender, n	
Male	2
Female	3
Mean age (±SD), yrs (range)	63 (±11), (54-83)
Mean storage time (±SD), days (range)	16 (±5), (8-23)
Mean ECD (±SD), cells/mm^2 (range)	2375 (±393), (1800-2900)
Cause of death, n	
Cardio/Stroke	4
Respiratory	1

Table 1: Demographics of donor data. *Mean storage time = time between death and culture of first isolated DM-EC tissue; SD = standard deviation; yrs = years

Isolation and Growth of Human Corneal Endothelial Cells

A two-step, peel-and-digest method was applied for primary hCEC isolation. The DM-EC sheets were first prepared as described previously[22,23] and stored in growth factor–depleted, DMEM-based medium for 4–7 d before isolation and expansion of the cells[24].

hCEC were isolated from the DM-EC sheets as described previously[24]. Briefly, the DM-EC sheets were exposed to 2 mg/ml collagenase (in DMEM) solution for 3–6 h at 37ºC and 5% CO_2 to dislodge hCEC from DM, which resulted in tightly packed hCEC clusters. The hCEC clusters were further dissociated into single cells with TrypLETM for 5 min at 37ºC and the resulting cell suspension was centrifuged at 500 rpm for 5 min at 37ºC. The cell pellet was resuspended in fresh culture medium (DMEM containing 15% FBS, 2 mM L-glutamine, 2

ng/ml FGF, 0.3 mM Asc-2P, and 100 IU/ml Pen/Strep Pre-Mix; supplemental data Table S1) and plated onto organ-tissue plates coated with FNC coating mix. Seeding cell density was determined by using 10 ml of cell suspension for an automatic cell count (Spark™ 10 M multi-mode microplate reader, Tecan Trading AG, Männedorf, Switzerland). The morphology of the cultured hCEC at con-fluence and during expansion was observed with an Axio-Vert.A1 microscope with AxioCam ERc 5s stand-alone functionality camera (Zeiss, Oberkochen, Germany). When reaching 80%–90% confluence (approximately after 3 wk), the culture medium was then replaced with stabilization medium (DMEM containing 15% FBS and 100 IU/ml Pen/Strep Pre-Mix; supplemental data Table S1) for the next 2–4 d before passaging based on the "dual media" approach described by Peh et al.[25], to enhance the cellular morphology of the expanded hCEC. Upon passaging, primary cultures were treated with 0.05% Trypsin/0.02% EDTA solution to allow detachment from the coated culture well plates and subcultured at a 1:2 splitting ratio on FNC-coated culture well plates. Before seeding cells onto the carriers (HALC, LK20, dDM), hCEC were passaged twice (up to P2) and cultured up to 1 wk on the carriers before using the hCEC–carrier constructs for the *in vitro* surgeries.

Cell Proliferation and Viability

Immunohistochemical staining of proliferating cells was determined with anti-Ki67 antibody[26] as previously described[27], followed by incubation with DAPI for staining nuclear deoxyribonucleic acid (DNA). Cell viability was measured using a ReadyProbes™ Cell Viability Imaging Kit. P2-cultured hCEC on FNC-coated substrates were incubated with the premixed solution of live and dead cell stain reagents for 15 min. The number of cell nuclei with com-promised plasma was determined and averaged in three areas, each measuring 0.01 mm^2, on digital microphotographs. Fixed-frame method was used to manually calculate the percentage of viable cells.

Preparation of Cell Carriers

HALC and dDM were isolated from human donor eyes at Amnitrans EyeBank Rotterdam, as previously described[22,23,27]. The HALC carriers were prepared by gently lifting the anterior capsule with forceps. An incision into the lens equator was made with a surgical blade to accomplish a 360ºcut. The obtained HALC were spread epithelial side up, and the lens cortex which was still adherent to the posterior side was removed using a surgical spear (Simovision BV, Diemen, the Netherlands) soaked in 70% ethanol. The HALC were then trephined to the desired size (Ø 8.0 mm) from the interior side. dDM was prepared by first isolating a DM-EC sheet as previously described and then by trephining the carrier to the customized size (Ø 8.0 mm). Both the isolated HALC and the DM-EC sheet were then treated with TE solution for 20 min to obtain a decellularized carrier (supplemental Figure S1). Remaining, loose lens epithelium or endothelium of the HALC and the DM, respectively, were removed by mechanical sweeping using a sponge soaked in balanced salt solution (BSS, Alcon Nederland BV, Gorinchem, the Netherlands).

For cell seeding, the freshly isolated HALC (with the epithelial side down)[17] and dDM (with the endothelial side up) were spread over FNC-coated glass coverslips in 48-well plates and kept moist in PBS. For both carriers, the orientation was carefully checked before spreading over the FNC-coated glass, in order to ensure the cell seeding on the correct side of the membrane (nonepithelial side for the HALC and endothelial side for the dDM).

LK20 were provided by LinkoCare Life Sciences AB (Linköping, Sweden). Diameter of the LK20 carriers for the experiments was 8.0 mm.

Immunofluorescence

For cell characterization, phenotypical markers ZO-1 and Na^+/K^+-ATPase were used[28,29]. Cells at P2 were cultured for 1 wk directly on the biocompatible carriers and were fixed with 4% PFA in PBS or ice-cold methanol for 15 min at room temperature. Next, samples were permeabilized using 0.1% Triton X-100 and unspecific binding of the anti-bodies was blocked with 3% BSA in PBS for 30 min. Samples were subsequently incubated with primary antibodies anti-ZO-

1/TJP1 (dilution 1:100) and anti-Na^+/K^+-ATPase (dilution 1:100) for 1 h, followed by secondary antibodies (dilution 1:200) in the dark for 45 min at room temperature. Both primary and secondary antibodies were diluted in blocking buffer and samples were rinsed with PBS before and after each incubation step. Further, the samples were stained with the DNA-specific dye DAPI, and then imaged using an inverted fluorescence microscope.

In Vitro Surgeries

In vitro surgeries were performed using human anterior corneal remnants (donor corneas of which the endothelium and attached DM had been peeled off) mounted onto an artificial anterior chamber (AC; DORC International, Zuidland, the Netherlands)[30]. Surgeries were performed by two experienced DMEK surgeons (ID, SND) who were masked regarding the used carrier to avoid bias.

In vitro surgical procedure was performed similar to a standard DMEK surgery[31] with some modifications as described previously[27]. In brief, three side ports were made at 2, 7, and 10 o'clock limbus-side, the artificial AC was filled with PBS, and a 3-mm main incision was made at 12 o'clock. After staining with 0.04% Trypan Blue, hCEC–carrier constructs were implanted through the main incision into the artificial AC by means of our standard DMEK glass injector (DORC International). Two of the constructs, the HALC and the dDM, were seen to roll with the endothelium facing inwards, which is the opposite direction to a standard DMEK roll. The surgery was therefore modified. The "Moutsouris sign" orientation[31] check was performed behind the roll rather than on top, and a small air bubble for unrolling was placed under the constructs, rather than over the hCEC–carrier constructs. Next, the hCEC–carrier constructs were centered, unfolded, and repositioned against the posterior stroma by using a larger air bubble. The AC was then fully pressurized with air for 1 h in order to enhance the adherence of the hCEC–carrier constructs to the posterior stroma. After 1 h, a partial air–fluid exchange was performed through the paracentesis, leaving a 50% air fill, and the hCEC–carrier construct attachment to the posterior stroma was evaluated by anterior-segment optical coherence tomography (AS-OCT; Heidelberg Engineering GmbH, Heidelberg, Germany). All hCEC–

carrier constructs were subjectively scored by the surgeon on a 5-point scale (i.e., 0–5) on the following parameters: (1) ability to roll in BSS, (2) staining intensity with 0.04% Trypan Blue solution, (3) insertion and positioning into the artificial AC, (4) response to surgical graft unfolding techniques, (5) transparency, and (6) ability to adhere to the posterior surface of the donor corneal stroma. Higher scores indicated closer resemblance to the DMEK reference model (i.e., DMEK control grafts).

Cell Viability After *In Vitro* Surgeries

Calcein-AM was applied to the hCEC–carrier constructs to assess endothelial cell viability after *in vitro* surgeries. Briefly, the hCEC–carrier constructs were carefully transferred endothelial side up from the human anterior corneal remnants onto silane-coated glass slides and incubated for 45 min in the dark at room temperature with 100 ml of PBS containing 400 mM Calcein-AM. After one more PBS washing step, fluorescence images of the hCEC–carrier constructs were taken, and the level of cellular fluorescence was deter-mined with ImageJ (National Institute of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/) using the thresholding method. In brief, the fluorescence images were converted to 8-bit images to measure the fraction of pixels (i.e., particle area fraction) in the selection that represents fluorescence intensities from viable cells.

RESULTS

hCEC Viability and Proliferation During *In Vitro* Expansion

The native corneal endothelium is a uniform monolayer of tightly packed, hexagonal CEC (**Figure 1A**) that express phenotypical and functional markers such as ZO-1 and Na^+/K^+-ATPase, respectively (**Figure 1 B, C**). Primary hCEC cultured and expanded *in vitro* formed a uniform cell layer with expression of ZO-1 and Na^+/K^+-ATPase (**Figure 1 D–F**). Moreover, cultured hCEC retained proliferative capacity (**Figure 2A**) and high cell viability (96% + 4%) (**Figure 2B**).

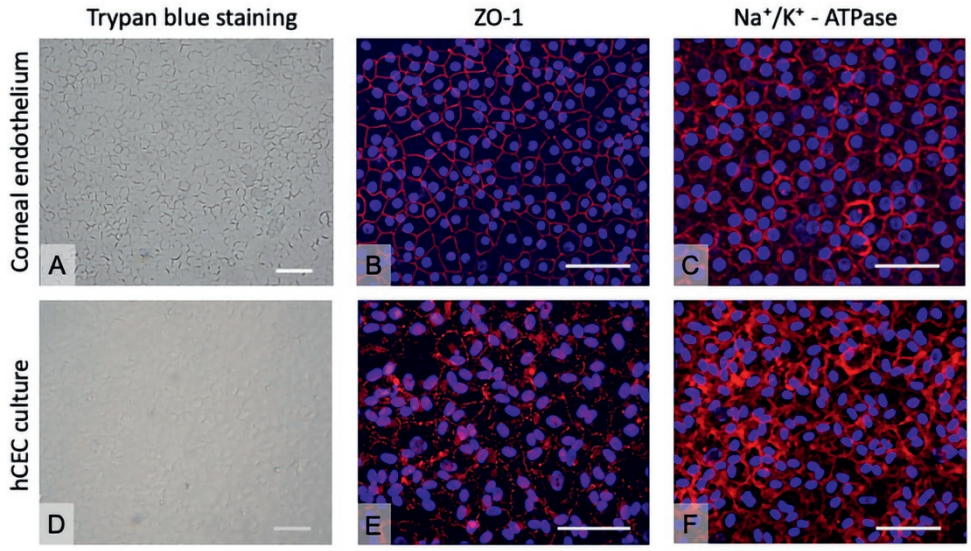

Figure 1: Evaluation of cell morphology on human corneas and of hCEC cultures. (A) Human cornea stained with hypotonic Trypan Blue solution 0.04%. (B) Expression of ZO-1 detected via immunofluorescence on human corneal endothelium. (C) Expression of Na^+/K^+-ATPase detected via immunofluorescence on human corneal endothelium. (D) hCEC cultured at P2 upon FNC-coated glass coverslips and stained with hypotonic Trypan Blue solution 0.04%. (E) Expression of ZO-1 detected via immunofluorescence on hCEC cultured at P2 upon FNC-coated glass coverslips. (F) Expression of Na^+/K^+-ATPase detected via immunofluorescence on hCEC cultured at P2 upon FNC-coated glass coverslips. Scale bars: 100 μm. ATP: adenosine triphosphate; FNC: fibronectin, collagen, and albumin; hCEC: human corneal endothelial cells; ZO: zonula occludens.

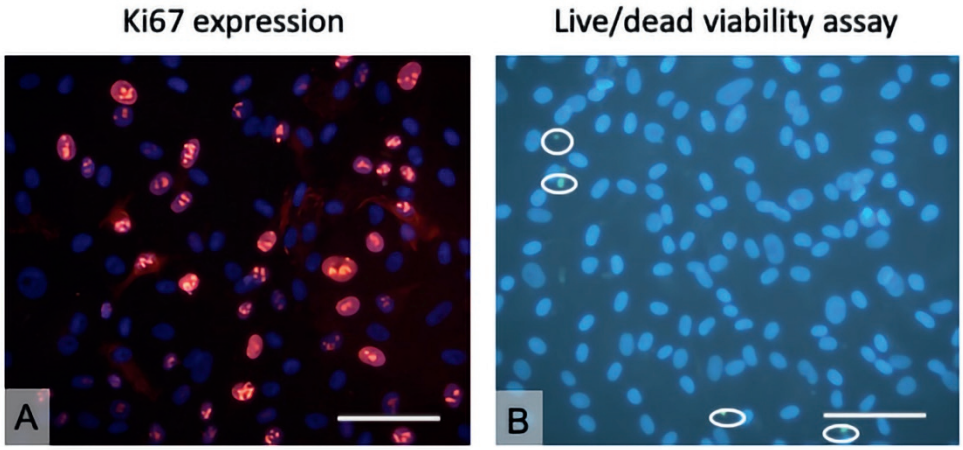

Figure 2: hCEC proliferation and viability at P2. (A) Evaluation of cell proliferation by expression of Ki67 (red). Nuclei are stained by DAPI in blue. (B) Live–dead assay to determine cell viability (blue: live; green, in circles: dead (= compromised plasma membrane integrity). Scale bars: 100 μm. DAPI: 40,6-diamidino-2-phenylindole; hCEC: human corneal endothelial cells.

hCEC–Carrier Constructs

Confluent cells at P2 were dissociated and seeded on the selected carriers (HALC, LK20, dDM) with an average cell seeding density of 36,932 ± 15,393 cells/ml of the cell suspension. At confluence, average densities were 2,033 ± 275, 1,911 ± 285, and 1,733 ± 258 cells/mm^2 for cells seeded on the HALC, LK20, and dDM carriers, respectively, with no significant difference between the cell density on the HALC and LK20 carriers, but lower densities on the dDM with cells displaying a flat and spread morphology (**Figure 3**). hCEC on HALC and LK20 showed unremarkable expression of the markers ZO-1 and Na^+/K^+-ATPase, while expression of Na^+/K^+-ATPase for cells seeded on the dDM was mainly cytoplasmic (**Figure 4**). After detaching the con-structs from the FNC-coated coverslips, the hCEC–HALC and hCEC–dDM constructs, floating freely in culture medium, showed the tendency to spontaneously form a roll, with the hCEC located on the inner surface, unlike a DMEK roll where the endothelium is located on the outer surface. Because of their biomechanical properties, hCEC–LK20 constructs did not form a roll in culture medium.

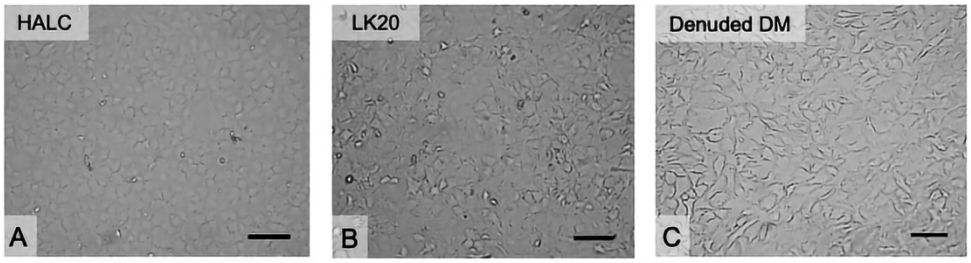

Figure 3: hCEC morphology at P2 on different biocompatible carriers. Series of illustrative figures showing hCEC cultures at P2 on different carrier. (A) HALC, (B) LK20, (C) denuded DM. Scale bars: 100 μm. DM: Descemet membrane; HALC: human anterior lens capsules; hCEC: human corneal endothelial cells; LK20: LinkCell™ bioengineered collagen sheets of 20-mm thickness.

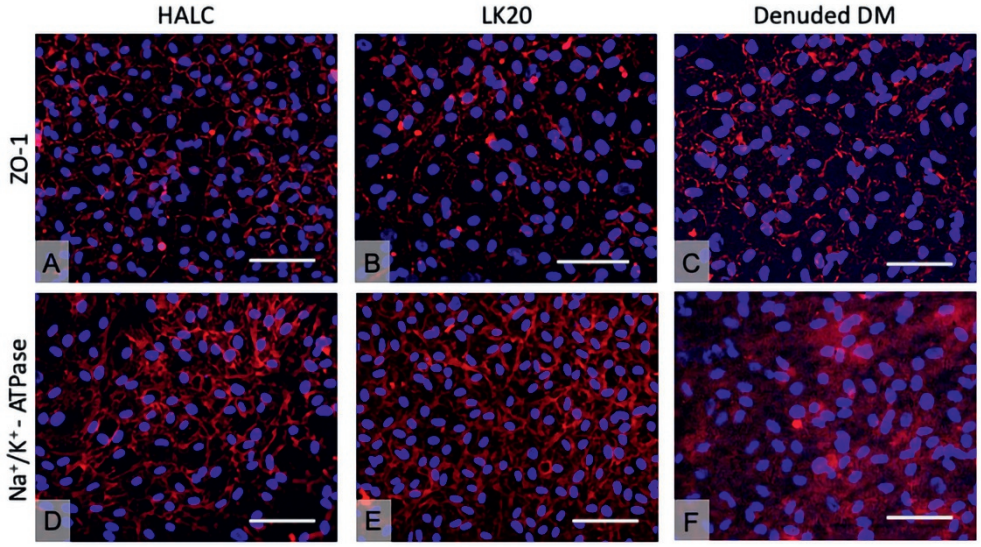

Figure 4: hCEC morphology check at P2 through immunofluorescence on different carriers. (A–C) ZO-1 expression detected in hCEC cultured on HALC, LK20, and denuded DM, respectively. (D–F) Na^+/K^+ expression detected in hCEC cultured on HALC, LK20, and denuded DM, respectively. Scale bars: 100 μm. ATP: adenosine triphosphate; DM: Descemet membrane; HALC: human anterior lens capsules; hCEC: human corneal endothelial cells; LK20: LinkCell™ bioengineered collagen sheets of 20-μm thickness; ZO: zonula occludens.

In Vitro Surgeries With hCEC–Carrier Constructs

In vitro surgeries were performed for the HALC (n = 5), LK20 (n = 4), and dDM (n = 3) cell–carrier constructs. Additionally, standard DMEK-grafts were used as a positive control (**Figure 5**) and served as a reference for the scoring (**Table 2**). The hCEC–HALC constructs behaved overall very similar to the DMEK reference model. These constructs could be stained with 0.04% Trypan Blue for visualization during surgery and loaded as "double rolls" in a DMEK glass injector (**Table 2, Figure 5 A, B**). While standard DMEK-grafts roll with their endothelium on the outer surface, hCEC–HALC constructs rolled with the hCEC on the inner side. Before implantation, the graft orientation was carefully checked under a surgical microscope with particular attention to position the endothelium in its correct orientation (**Figure 5B**). All hCEC–HALC constructs could be implanted and unfolded by using a surgical approach similar to a DMEK procedure. Unfolding and positioning of the con-structs against the posterior surface of the stroma was achieved in a "no-touch" fashion, with some modification to the technique as described earlier (**Figure 5C**). One-hour post surgery, OCT measurements showed almost complete adhesion to the bare stroma after a partial air–fluid exchange (**Figure 5D**).

The hCEC–LK20 constructs did not roll spontaneously but did show a similar staining capacity with Trypan Blue when compared to the hCEC–HALC constructs and standard DMEK-grafts (**Table 2, Figure 5E**). In order to inject the con-struct, a cannula was used to lift and manually load the hCEC–LK20 construct into the injector, with the endothelium on the external surface (**Figure 5F**). The unfolding of the hCEC–LK20 construct in the artificial AC resembled the unrolling of a Descemet stripping automated endothelial keratoplasty graft more than that of a DMEK but the transparency was comparable to the other two tested carriers (**Figure 5G**). The graft was lifted to the posterior stroma, but the rigidity of the material resulted in some radial peripheral fold and OCT measurement revealed incomplete adherence of hCEC–LK20 constructs to the bare stroma, 1-h post *in vitro* surgery (**Figure 5H**).

The hCEC–dDM constructs behaved in a manner similar to the hCEC–HALC constructs in both Trypan Blue staining (**Figure 5I**) and rolling in BSS (with its endothelium on the inside of the scroll). Although these constructs tended to be

fragile and slippery when loaded into the injector, the implantation in the artificial AC and the unfolding were successfully achieved in all cases (**Figure 5 J, K**). One-hour post *in vitro* surgery, all hCEC–dDM constructs showed good adherence to the bare stroma (**Figure 5L**).

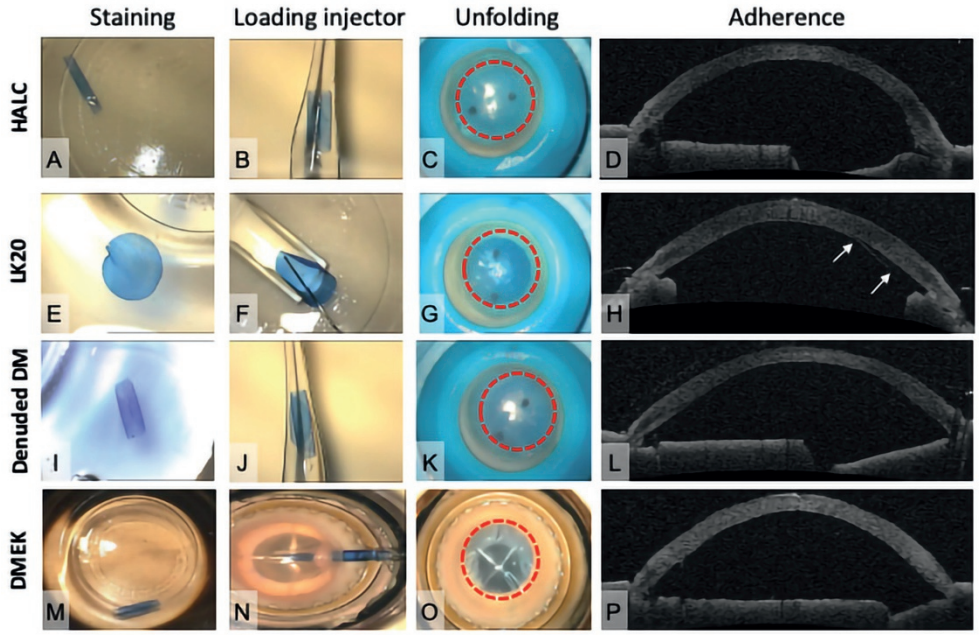

Figure 5: *In vitro* surgeries with hCEC–carrier constructs. The upper three rows show each of the three tested hCEC–carrier constructs after staining with hypotonic Trypan Blue solution 0.04% (A, E, I), loading into the injector (B, F, J), insertion into the artificial anterior chamber and unfolding (C, G, K), and adherence to the bare stroma visualized by anterior segment OCT measurement (D, H, L). The lower row (M–P) shows the corresponding images of a DMEK-graft that served as a positive control and reference point for the scoring. White arrows in image (H) indicate the area without adherence to the bare stroma. DM: Descemet membrane; DMEK: Descemet membrane endothelial keratoplasty; HALC: human anterior lens capsules; hCEC: human corneal endothelial cells; LK20: LinkCell™ bioengineered collagen sheets of 20-µm thickness; OCT: optical coherence tomography.

Carrier type	DMEK-graft (positive control)	HALC	LK20	Denuded DM
Rolling in BSS	5	4.3 (±1.2)	0.7 (±0.3)	4 (±0.5)
Staining	5	3.7 (±1.0)	4.3 (±0.6)	3.7 (±1.0)
Anterior chamber Implantation	5	4.3 (±0.3) (similar to DMEK, easy loading)	4.0 (±0.0) (manipulation required to obtain DMEK-roll)	4.5 (±0.0) (similar to DMEK, 'slippery' when loaded into the injector)
Protocol followed	DMEK	DMEK	DMEK	DMEK
Unfolding	5	4.5 (±0.0) (similar to DMEK, easily centered)	4.5 (±0.5) (similar to DMEK, easily centered)	4.5 (±0.0) (similar to DMEK, easily centered)
Transparency	5	4.7 (±0.3)	4.7 (±0.6)	4.7 (±0.3)
Adherence	5	4.2 (±0.8)	2.2 (±0.8)	4.3 (±1.2)

Table 2: *In Vitro* Surgeries With hCEC–Carrier Constructs: Scoring Scheme. Each parameter under evaluation was rated on a 5-point scale (i.e., 0–5), with higher score indicating greater similarity to *in vivo* DMEK behavior. DMEK-grafts were tested as a positive control and served as a reference point for the scoring. Values are reported as mean (+ standard deviation). BSS: balanced salt solution; DM: Descemet membrane; DMEK: Descemet membrane endothelial keratoplasty; HALC: human anterior lens capsules; LK20: LinkCell™ bioengineered collagen sheets of 20-µm thickness.

After the OCT measurements, endothelial cell morphology and viability were evaluated on reference DMEK-grafts, hCEC–HALC, and hCEC–LK20 constructs. Calcein-AM staining showed minimal damage to the reference DMEK-graft (viability of 90 (±3)%) except for some areas that were already affected after graft preparation due to the low quality of the cornea that had been discarded for transplantation (**Figure 6 A, D**). Light microscopy showed

centrally an almost intact monolayer of hexagonal hCEC on the HALC (**Figure 6B**), while the hCEC layer on the LK20 showed several gaps (**Figure 6C**). This finding was also reflected by Calcein-AM staining which resulted in an estimation of ~83 (±5)% and ~67 (±9)% of viable cells present after *in vitro* surgery on hCEC–HALC constructs and hCEC–LK20 constructs, respectively (**Figure 6 E, F**).

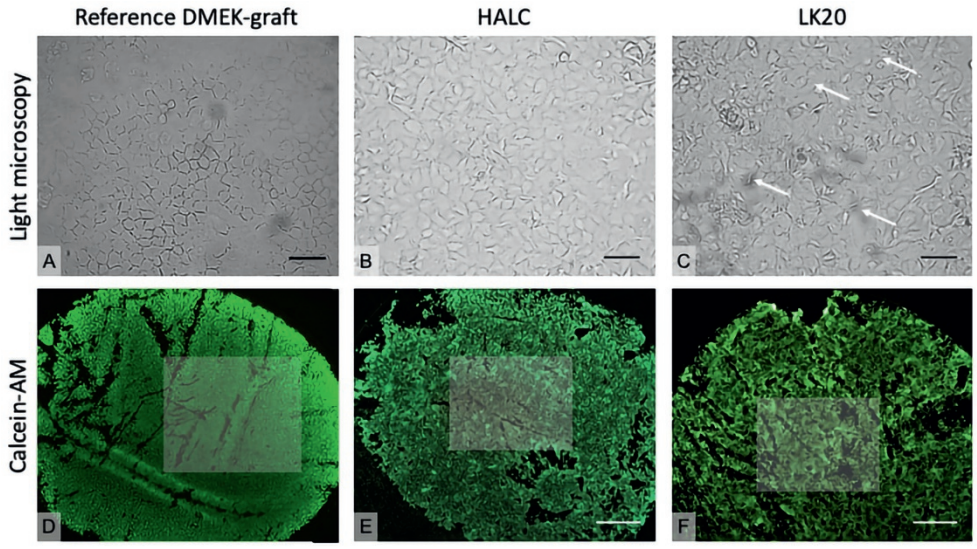

Figure 6: Cell assessment after *in vitro* surgeries. (A–C) Central light microscopy images of a DMEK reference graft and the hCEC–HALC and hCEC–LK20 constructs. White arrows in (C) point to gaps in the hCEC monolayer. Scale bars = 100 µm. (D–F) Calcein-AM expression detected on the DMEK reference graft and the hCEC–HALC and hCEC–LK20 constructs, respectively. Scale bars = 1 mm. DMEK: Descemet membrane endothelial keratoplasty; HALC: human anterior lens capsules; hCEC: human corneal endothelial cells; LK20: LinkCell™ bioengineered collagen sheets of 20-µm thickness.

DISCUSSION

In this comparative study, we tested hCEC–carrier constructs in *in vitro* surgeries comparing them to a DMEK-graft reference model in terms of rolling, staining, unfolding, and adherence to the bare posterior stroma of a human donor anterior remnant.

Tissue-engineering of corneal grafts by *in vitro* expansion of hCEC relies on the availability of suitable biocompatible carriers. Ideal carrier candidates, which could be of different origins ("biological" and "bioengineered"), composition, and biomechanical properties, should mimic native tissue microenvironment to assure cellular functionality and offer structural and mechanical support for the transplantation using the DMEK technique.

Our study confirmed the potential of HALC as a cell culture substrate as previously shown for different types of ocular cells[32–34], including hCEC[15,17]. Its composition is one of the major advantages of the HALC as a scaffold, as it is very similar to the native DM. HALC is mainly formed by the membrane protein collagen IV and other matrix components including collagen I, collagen III, collagen VIII, laminin, and fibronectin[35]. These core molecules self-assemble into a 3D matrix that provides strength and flexibility to the lens. During *in vitro* surgery, hCEC–HALC constructs behaved comparable to the DMEK reference model in terms of loading into the artificial AC and adherence to the stroma even though the hCEC–HALC constructs showed a "reversed" rolling with the endothelial cells on the inside. After *in vitro* surgeries, hCEC–HALC constructs also retained a high endothelial cell viability rate (~82% of the surface area) suggesting that these constructs may be handled surgically with minimal effect on cell viability.

The second biological construct, hCEC–dDM also showed similarities with the DMEK reference model in terms of staining and adherence upon *in vitro* surgery, which may be related to similar biological properties shared by the two carriers. Seeding of the cells on the dDM carriers induced a "reversed" rolling of the constructs, that is, with the endothelial cells on the inside, as also observed for the hCEC–HALC constructs. Therefore, hCEC–HALC and hCEC–dDM constructs had to be implanted into the artificial chamber by providing small changes compared to the standard DMEK surgical technique. However, in the

course of the study there were several issues with the dDM carriers, which we may have not described explicitly enough in the first manuscript draft and which led to the decision not to perform tests on the cell viability.

The preparation of dDM requires an enzymatic treatment in order to remove all native CEC present on the DM prior to using it as a cell culture carrier. This enzymatic treatment has been reported to also remove parts of the fibronectin-mediated cell–extracellular matrix interaction[36]. Alternatively, usage of a cotton swab to mechanically disrupt cells might create microgrooves subsequently affecting cell alignment and morphology of the freshly seeded CEC[36]. In addition, dDM originating from elderly donors shows a highly structured surface due to imprints left by the removed cells. This structured surface seems to impair the morphology of the cultured cells on these carriers.

In this early stage of developing tissue-engineered cell–carrier constructs as a potential alternative to standard endothelial grafts, both of the tested natural carriers can serve as a benchmark for bioengineered carriers in terms of required surgical handling and robustness upon surgical manipulation in an *in vitro* setting. For potential future clinical applications, however, using natural carriers such as HALC and dDM as a substrate will still be dependent on donor tissue.

An alternative to the natural carriers is bioengineered substrates such as the LK20 carrier. Bioengineered materials offer distinct advantages over natural carriers: they are donor-independent and could be produced in large quantities and with reproducible quality. Previous studies reported, for example, on the use of silk fibroin-based carriers or plastic compressed type I collagen hydrogel membranes (termed RAFTs)[37,38] as cell carriers and we also reported the feasibility of collagen I–based carrier LK20 for culturing and expanding porcine CEC[27]. Here, we have shown that hCEC could also be successfully cultured on the LK20 carrier. However, upon *in vitro* surgery, the hCEC–LK20 constructs showed less adherence to the posterior stroma than other carriers after implantation. In addition, an endothelial cell viability rate of ~67% after *in vitro* surgery may either reflect cell damage due to the additional handling required during the surgery or may be due to a "compatibility issue" between cells and carrier. In a study involving hCEC seeding on fish scale–derived scaffolds, it has been reported that the stiffness of the underlying matrix (expressed as Young's

modulus) was not sufficient for the cells to properly adhere to the carriers[39]. Improvements on the biophysical (strength of cell-substrate adhesion or membrane permeability) and biomechanical properties (e.g., tensile strength, elasticity) may facilitate cell adherence to the LK20 carriers as well as the surgical handling, which should be further validated *in vitro* to define the potential of LK20 collagen sheets as suit-able carriers for hCEC sheet transplantation.

Some limitations of this study have to be highlighted such as the small sample size. Due to the limited availability of research corneas of sufficient quality, tests could not be performed on a large scale to improve repeatability and reproducibility. In addition, donor age of the available donor cornea, which is mainly derived from elderly donors, may affect the hCEC proliferation capacity. Although there was no statistical difference in donor age for cells seeded onto different carriers, observed hCEC morphology may still have been affected by donor age.

In conclusion, all selected carriers were suitable for *in vitro* hCEC culture and expansion, and *in vitro* surgical manipulation showed that the hCEC–carrier constructs behaved mostly like the DMEK reference model, especially the hCEC–HALC construct. However, for potential clinical applications, the use of natural carriers would require reproducible carrier quality while biomechanical properties of the tested bioengineered carrier would need to be further improved to ensure successful graft function.

ETHICAL APPROVAL

Ethical approval is not applicable for this article as under national regulation no approval is required for this research if no extra procedure was performed to obtain the samples, and donors had consented to having the samples used for research purposes (https://www.ccmo.nl/onderzoekers/soorten-onderzoek/niet-wmo-onderzoek/onderzoek-met-lichaamsmateriaal).

STATEMENT OF HUMAN AND ANIMAL RIGHTS

This article does not contain any studies with human or animal subjects.

STATEMENT OF INFORMED CONSENT

There are no human subjects in this article and informed consent is not applicable.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Dr Melles is a consultant for DORC International/Dutch Ophthalmic USA and SurgiCube International. Dr Rafat serves on the Board of Directors of LinkoCare Life Sciences AB, which is a spin-off firm developing products related to the research being reported and holds relevant patents. Dr Rafat's terms of arrangements have been reviewed and approved by Linköping University in accordance with its policy on objectivity in research. The other authors have no conflicting relationship to disclose.

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SUPPLEMENTARY MATERIALS

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CHAPTER 7

Adapted from:

Göttingen Minipig is not a Suitable Animal Model for *in Vivo* Testing of Tissue-Engineered Corneal Endothelial Cell-Carrier Sheets and for Endothelial Keratoplasty

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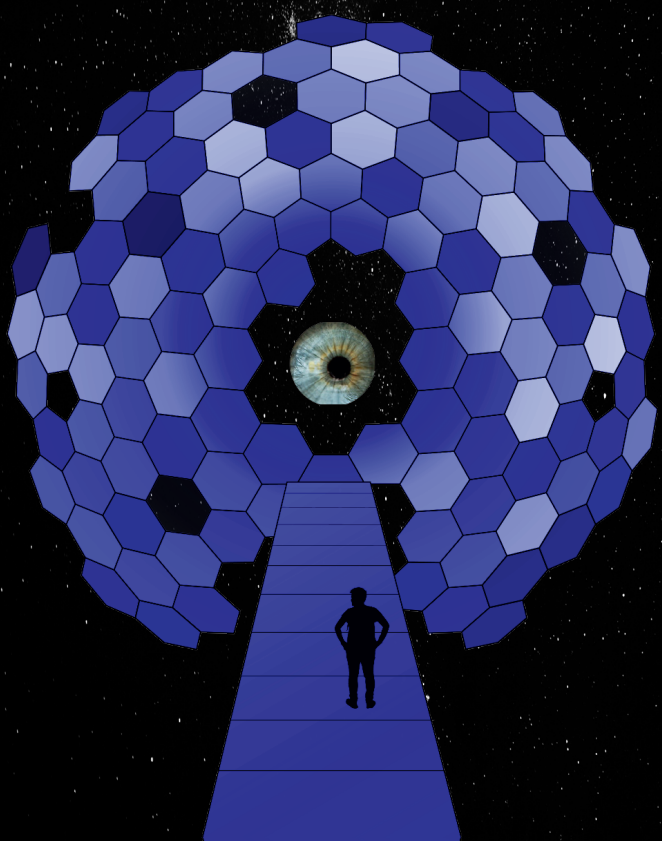

ABSTRACT

Aim: To test the feasibility of implanting human anterior lens capsules (HALCs) with porcine corneal endothelial cells (pCEC) *in vivo* in Göttingen minipigs and at the same time test the suitability of Göttingen minipig as model for endothelial keratoplasty.

Materials and Methods: Cell-carrier constructs of decellularized HALC with cultured (pCEC) were created for implementation *in vivo*. Eight Göttingen minipigs (6 months old) underwent surgery with descemetorhexis or removal of endothelium by scraping and implementation of HALC without (animal 1–4) and with (animal 5–8) pCEC. Follow-up examinations included optical coherence tomography (OCT) imaging (1, 2 and 3 months) and slit-lamp examination (<1 week as well as 1, 2 and 3 months).

Results: Intraoperative challenges included difficulties in maintaining an anterior chamber due to soft tissue and vitreous pressure, development of corneal edema and difficulties removing Descemet's membrane because of strong adhesion to stroma. Therefore, descemetorhexis was replaced by mechanical scraping of the endothelium in animal 4–8. HALCs without pCEC were implanted in animal 1–4. Apposition to the back surface was not achieved in animal 1 and 3 because of corneal edema and poor visibility. Animal 5 was sacrificed because of a lens capsule tear. HALCs with pCEC were implanted in animal 6–8. Slit-lamp examination the first week revealed corneal edema in all animals, although mild in animals 4. One-month examination showed retrocorneal membranes with overlying corneal edema in all animals. Histology showed fibrosis in the AC and on the back surface of the cornea, compatible with the clinical diagnosis of retrocorneal membrane.

Conclusions: In conclusion, the minipig is not suitable for corneal transplantation studies *in vivo* because of intraoperative challenges and development of retrocorneal membrane postoperatively. For *in vivo* testing of the surgical handling and the therapeutic potential of tissue-engineered endothelial cell-carrier constructs other animal models are required.

KEYWORDS: endothelial cell transplantation, cell carriers, animal models, retrocorneal membrane

INTRODUCTION

Endothelial keratoplasty is the most common treatment for corneal endothelial diseases, but worldwide shortage of donor corneas limits the number of transplantations that can be performed.[1] Tissue-engineered cornea analogs may provide an alternative to scarce human donor tissue,[2] but require *in vivo* testing in animals before introducing them into clinical practice. Both monkeys and rabbits have previously been used for this purpose,[3,4] while pigs have been used for implantation of collagen inlays and testing of fish scale-derived biocorneas.[5] Using pigs for preclinical studies is appealing because of the similarities between eyes in pigs and humans in terms of ocular anatomy (i.e. similar sized globes), intraocular physiology (vitreous humor properties) and vision and associated biometrics, and a greater availability compared with monkeys.

Several corneal endothelial cell-carrier constructs were recently evaluated by Spinozzi et al. in terms of surgical *in vitro* handling and isolated human anterior lens capsules (HALC) proved to be the most suitable carrier in that study.[6] The purpose of this study was to test the feasibility of implanting tissue engineered cell-carrier constructs (HALC with porcine corneal endothelial cells (pCEC)) *in vivo* in an animal model and test the suitability of Göttingen minipigs as a model for endothelial keratoplasty.

MATERIALS AND METHODS

Sample preparation

Isolation of pCECs was performed at Netherlands Institute for Innovative Ocular Surgery (NIIOS) as described previously.[6] After 2 passages, primary pCEC were seeded onto decellularized HALC. After cells reached confluence on the HALC carrier, samples were shipped to Aarhus in stabilization medium.[6,7]

Animal surgery

Ethical approval was obtained from the Danish Animal Experiments Inspectorate (2017-15-0201-01344). Surgery was performed in 8 six-month-old Göttingen Minipigs (Ellegaard, Denmark), one eye per pig, under general anesthesia at Aarhus University's animal facility Påskehøjgaard by an experienced cornea surgeon (JH). Preoperative baseline conditions were characterized by anterior segment optical coherence tomography (OCT) imaging (Telesto 2, Thorlabs). Surgical access to the anterior chamber (AC) was established via peripheral corneal incisions. Endothelium was removed either by descemetorhexis with a reversed Sinskey hook or mechanical scraping; both supported by either an AC maintainer (≈50cmH20) or an air-filled AC. HALCs were stained with trypan blue and injected with either an IOL-injector or a DMEK EndoGlide. After injection of the lens capsule into the AC, unfolding and positioning of the lens capsule against the posterior stroma were attempted. If unsuccessful, the lens capsule was left free-floating in the AC. Intracameral cefuroxime and subconjunctival triamcinolone was administered at the end of the procedure and the AC filled with a 20% SF6 gas-air mixture.

Follow up examinations

All animals underwent handheld (Kowa SL-17) slit-lamp examination (SLE) on day 3–7 as well as a SLE and OCT imaging in general anesthesia at 1 month, 2 months (animal 1–4) and 3 months (animal 1–3).

Histology

The eyes were harvested after the animals were sacrificed and corneal-scleral rims were excised and fixed in 4% formaldehyde. The specimens were cut in parallel slices, paraffin-embedded, sectioned and stained with either hematoxylin-eosin (HE), Weigert's elastic Van Gieson or periodic acid-Schiff (PAS) stain.

RESULTS

Animal surgery

Maintaining an AC proved difficult in all minipigs due to vitreous pressure and a rather soft cornea resulting in loose incisions. Descemetorhexis was attempted and achieved to some extent in minipigs 1–3. Descemet membrane was highly adherent to the stroma which complicated and prolonged the procedure, often resulting in corneal edema and therefore the endothelium was scraped off in the remaining animals (4–8). Minipig 5 was sacrificed immediately due to damage to the lens during surgery. Implementation of HALCs without pCEC was successful in minipigs 2 and 4, but unsuccessful in minipigs 1 and 3 because of poor visibility due to corneal edema and the HALC was left free-floating in the AC. HALCs with pCEC were successfully positioned onto the posterior stroma in minipigs 6–8, however, in minipig 6 the pCEC-HALC construct was placed upside-down with cells facing the posterior stroma.

Follow up examinations

Animal welfare was good in all animals during the study period as monitored by the animal caretakers.

SLE of all animals at day 3–7 revealed corneal edema, although in minipig 4 it was very mild. At one month all animals had developed a central retrocorneal membrane with overlying corneal edema (**Figure 1**). The only exception was minipig 4 which had a clear central cornea but formation of a peripheral retrocorneal membrane around the main incision. Anterior synechiae were present in minipigs 1–3.

OCT imaging at one month confirmed the presence of retrocorneal membranes in all animals (**Figure 2**). Although in minipig 4 it was less pronounced with only a small membrane located around an incision. In all eyes, the retrocorneal membranes were more prominent around the incisions. The central corneal thickness increased with >50% in all animals except minipig 4 (**Figure 3**).

Apposition of HALC was difficult to confidently deter-mine by OCT imaging as they were presumably covered by retrocorneal membranes. In minipig 4 the bare HALC had detached.

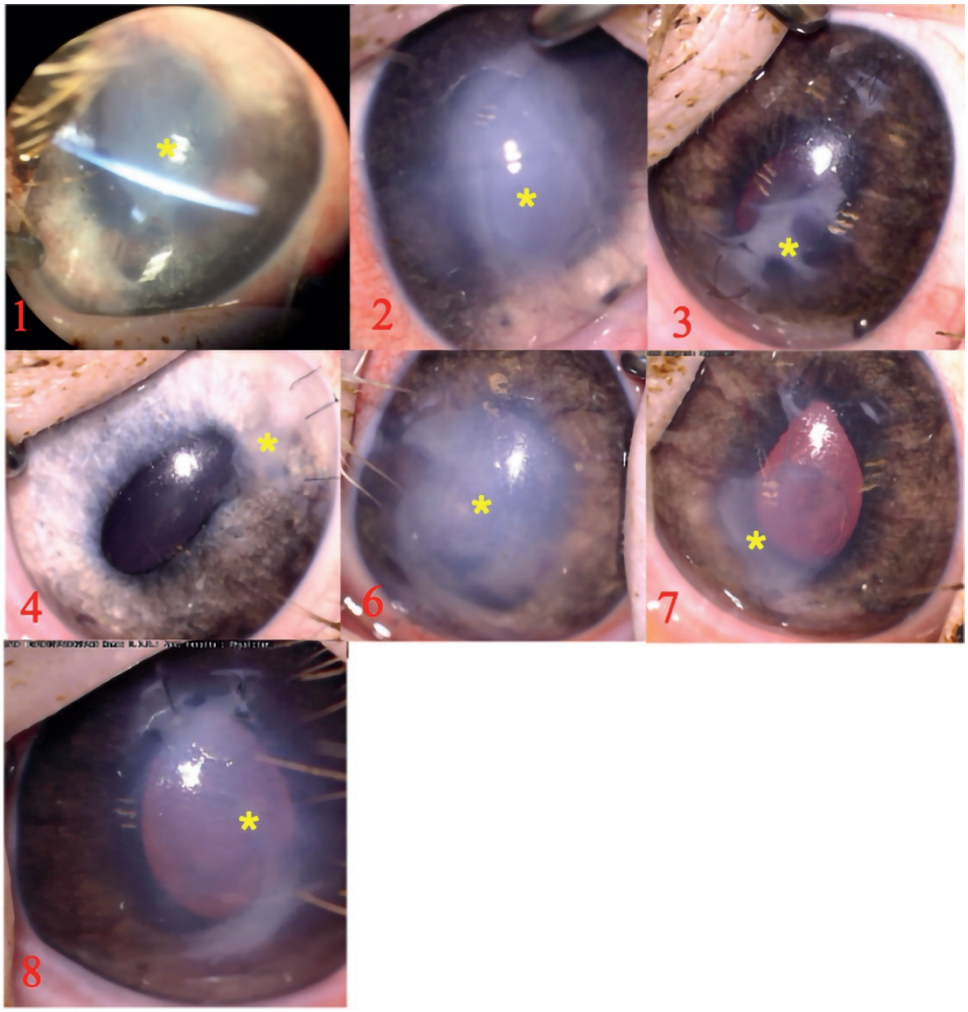

Figure 1: Photography at one month showing retrocorneal membrane/fibrosis formation in all eyes (yellow asterisk). In minipig 4 there is only a small membrane located around the main incision.

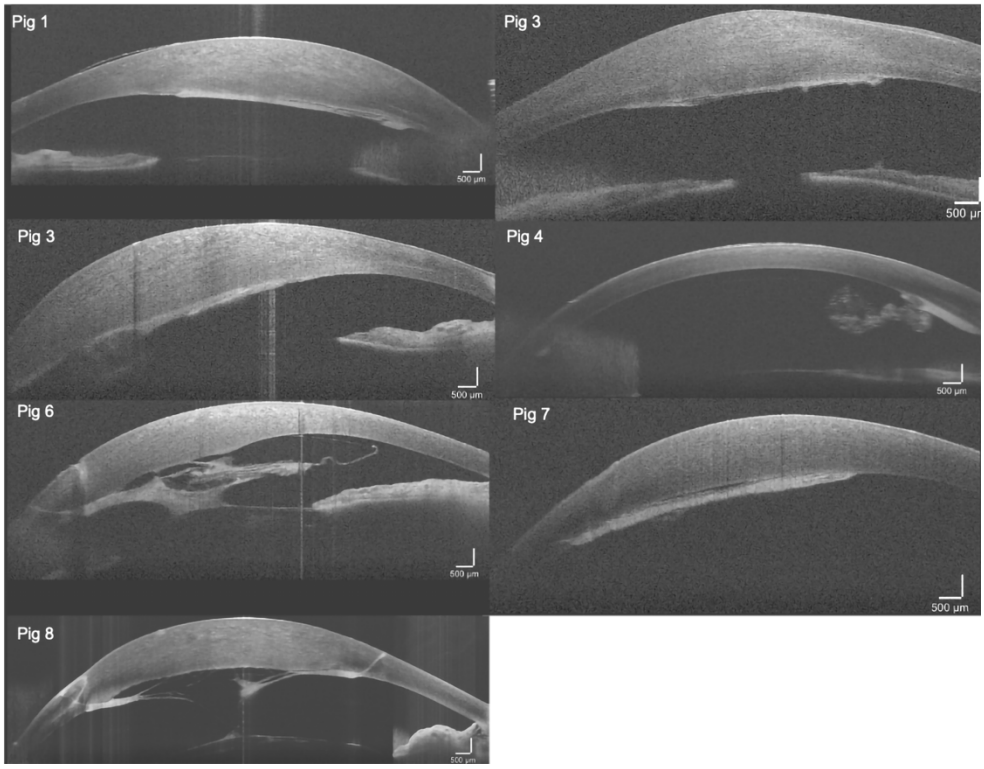

Figure 2: OCT imaging at one month showing retrocorneal membrane/fibrosis in all minipigs. Corneal edema was present in association with the membrane in all minipigs except for minipig 4, where retrocorneal membrane formation was confined to the area around the main incision. In minipigs 6 and 8 the membrane formed a web-like structure in the anterior chamber with adhesion to iris and lens.

Histology

Histology revealed that descemetorhexis (minipigs 1–3) induced a fibrotic response with associated angiogenesis. Scraping of the endothelium without persistent apposition of the HALC against the posterior cornea (minipig 4) resulted in minor localized fibrosis. The scraped area was covered by endothelial cells (**Figure 4**) and showed no obvious difference in cell count and morphology compared to control (minipig 3, non-operated eye). Some fibrosis on the posterior surface and in the AC was present in animals (6–8) that had the endothelium removed by scraping and a HALC successfully positioned against the posterior cornea. Examination of the interface between HALC and posterior

cornea revealed signs of a reactive response with cellular infiltration that included both fibroblasts but also other cells of unknown identity that might be transformed endothelial cells. There were endothelial cells remaining on the lens capsules but the numbers appeared sparse.

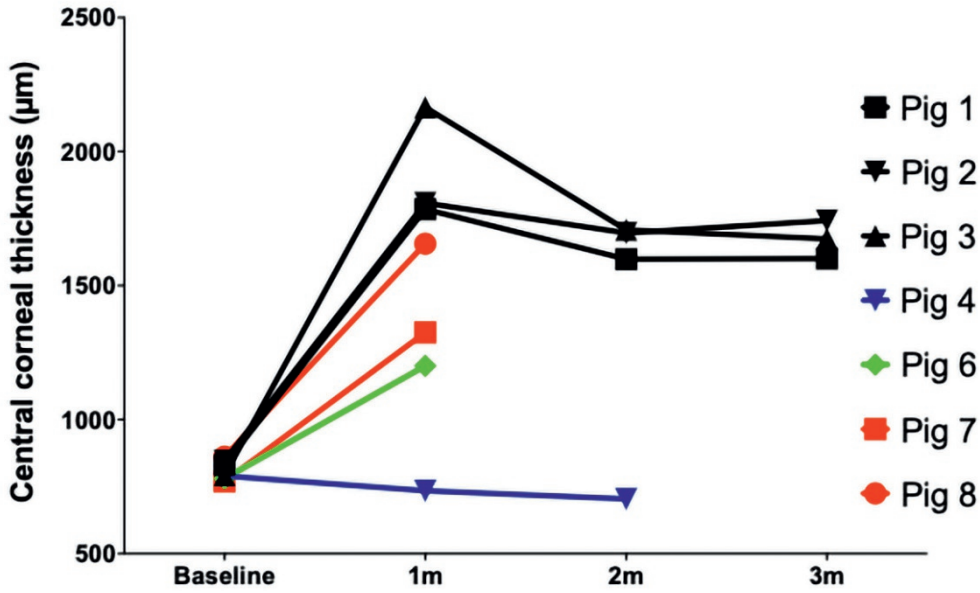

Figure 3: Central corneal thickness (µm) in all minipigs before and after surgery. The animals are color coded according to procedure and outcome. Black: descemetorhexis and implantation of lens capsule without endothelial cells. Blue: scraped endothelium with detached capsule without endothelial cells. Green: scraped endothelium and lens capsule with endothelial cells but positioned upside-down. Red: scraped endothelium and lens capsule placed correctly with endothelial cells facing the anterior chamber. Follow-up examinations were reduced in animals 4–8 due to minimal clinical changes after one month in the first cohort of animals undergoing surgery (animals 1–3).

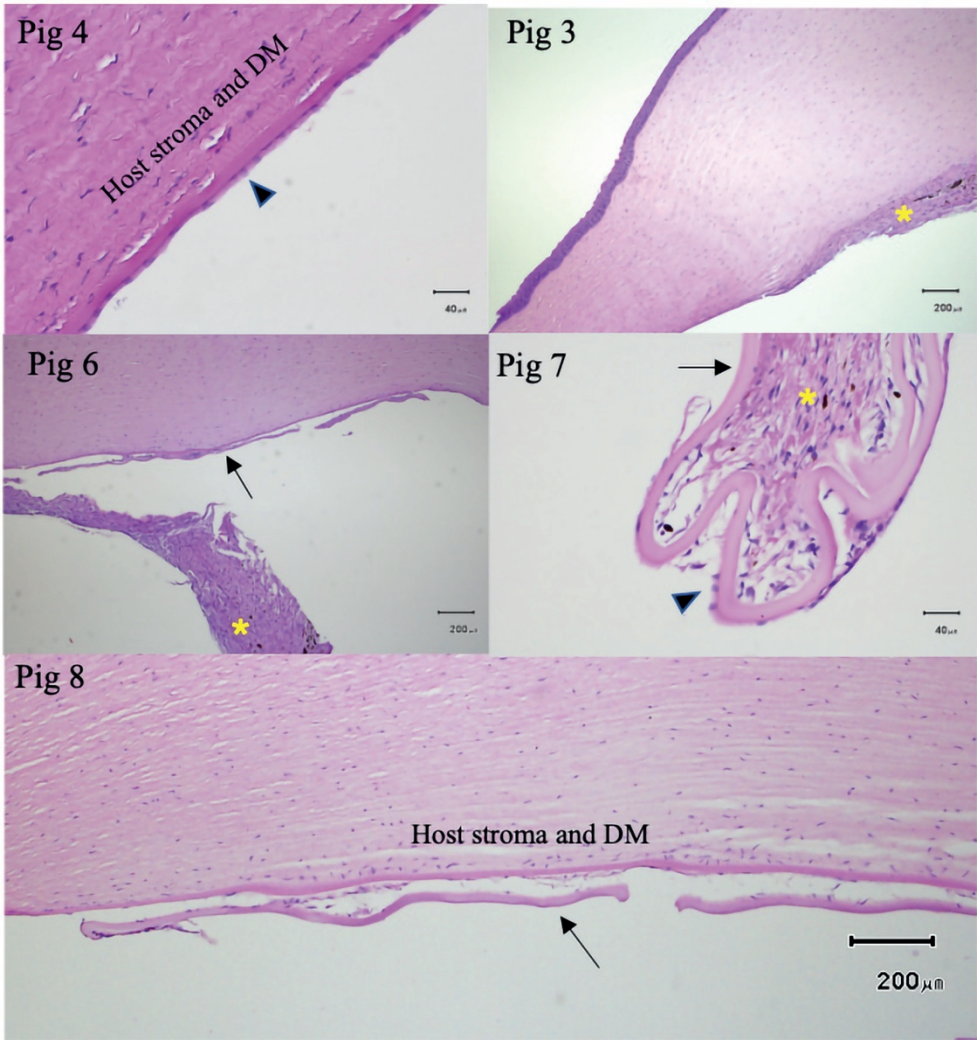

Figure 4: Examples of histological findings. Top left: One month after endothelial removal in minipig 4 by scraping histology revealed endothelial cells present in the same area. Top right: Minipig 3 showed fibrosis with angiogenesis on the posterior cornea after descemetorhexis. Middle left: Fibrosis in the anterior chamber (minipig 6). Middle right: Fibrotic reaction around a partially detached HALC (minipig 7). Endothelial cells can be seen on the HALC. Bottom: Reactive changes and cellular infiltration between HALC and host cornea. No endothelial cells can be seen on the HALC (minipig 8). Arrow head = endothelial cells, arrow = human anterior lens capsule (HALC), asterisk = fibrosis DM = Descemet's membrane.

DISCUSSION

The aim of this study was to test a tissue-engineered endothelial cell-carrier construct *in vivo* and to test the suitability of the minipig as a model for endothelial keratoplasty. Owing to the challenges encountered with using the minipig, the main outcome of the study regards the suitability of the minipig for transplant-related corneal research.

Our study present intraoperative and postoperative challenges with the minipig which makes it a less ideal model for endothelial keratoplasty. Descemetorhexis is routinely done in humans, although remnants can remain and may pose a risk for graft detachment.[8] In the minipig descemetorhexis was difficult to perform, most likely due to the young age of the animals. Therefore, the central endothelium was removed by scraping in four animals. The two procedures, however, seem to result in different outcomes: minipig 1 (descemetorhexis) developed a central retrocorneal membrane and corneal edema whereas minipig 4 (scraping) had a clear cornea with only a minor retrocorneal membrane around the main incision and evenly distributed endothelial cells on histology. This suggests that, contrary to primates,[4] pCEC can proliferate *in vivo* since it is unlikely that migration of remaining endothelial cells can compensate for such a large loss of cells.

Retrocorneal membranes developed in experiments with HALCs with and without pCEC, suggesting that it is not a pure rejection response to the transplanted endothelial cells. Retrocorneal membrane formation seemed to originate from the corneal incisions and may develop via either (1) epithelial downgrowth (2) fibroblastic or stromal (keratocytic) downgrowth, or (3) fibrous metaplasia of the corneal endothelium.[9] Any of these mechanisms may be involved here, though stromal keratocytes likely play the major role since the retrocorneal membrane formation seemed to originate from areas where the stroma had been traumatized, i.e. from the corneal incisions or over the descemetorhexis area. Recently, a study[10] demonstrated that in pig-to-monkey penetrating keratoplasty all monkeys developed retrocorneal membranes due to activation of stromal keratocytes in the pig graft. These findings also suggest that the pig/minipig is prone to developing retrocorneal membranes and that this limits the use of pigs/minipigs for corneal surgery studies. Regarding the cell-

carrier constructs, information obtained from this study was limited by the surgical difficulties and the strong wound-healing reaction. However, it was possible to sufficiently stain the cell-carrier constructs for visualization, inject them into the anterior chamber and position them against the posterior stroma. Histology images suggest a low number of retained pCEC on the HALC carrier after one month, possibly because of the surgical trauma and/or retro-corneal membrane formation. In addition, it suggests that host CEC did not proliferate/migrate onto the HALCs.

Endothelial keratoplasty is target for substantial research interest and there is a need for a good animal model. Many of the intraoperative challenges that can occur in a new model can likely be handled by alterations of the techniques used, but generating results with translational potential is important. Therefore, we suggest three criteria for a good model; eye bulb size similar to humans, removable descemet's membrane and non-proliferative CECs. Monkeys fulfil these criteria but are limited by accessibility. Rabbits are likely the best option. Although rabbits have proliferative CEC this may not be a problem as long as study duration is not too long (< 1 month) and graft CEC are labelled (e.g. DiI).

In conclusion, the minipig is not an ideal animal model for *in vivo* testing of tissue-engineered endothelial cell-carrier constructs and for endothelial keratoplasty studies in general. More research should be directed towards a better option for animal model, in terms of easiness in removing the descemet's membrane and eye bulb size similar to humans.

DECLARATION OF INTEREST

No conflict of interest.

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CHAPTER 8

Discussion and Summary

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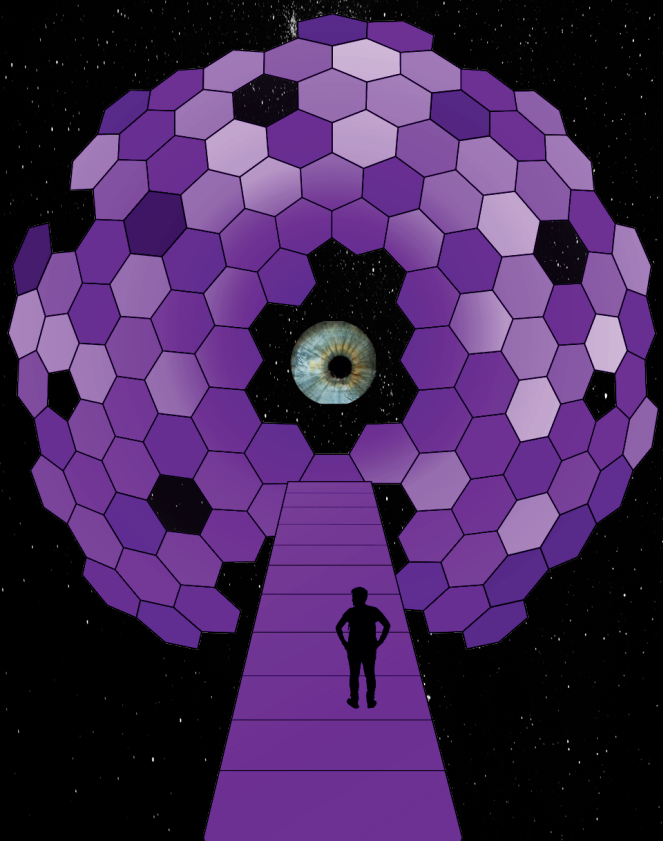

DISCUSSION AND SUMMARY

The development of Descemet's membrane endothelial keratoplasty (DMEK) represents a milestone in the history of corneal transplantation and, to date, DMEK is the most selective surgical technique to treat corneal endothelial pathologies, as it permits the replacement of the damaged corneal endothelium with its healthy counterpart from a donor.[1] Nevertheless, DMEK is strictly linked to the availability of donor tissue.

To overcome the dependence on donor tissue, given its global shortage, new cell-based approaches have been developed, in parallel with surgical approaches focusing on optimizing the use of donor endothelial grafts. The latter option is still preferred at the moment, given the high costs of the cell-based approaches. However, once more developments will be accomplished in the field of the cell-based therapies, it is reasonable to think that this branch will quickly become the preferred choice of treatment. The cell-based strategies rely on the *in vitro* expansion and culture of human corneal endothelial cells (hCEC), which reside on the posterior part of the cornea and form a single cell layer, which is responsible for the preservation of corneal hydration and corneal transparency.[2] As hCEC show a lack in proliferation *in vivo* as they are arrested in the G1 phase of the cell cycle,[3-5] endothelial cell death or dysfunction may eventually lead to a decrease or loss in corneal transparency. As loss of function of the corneal endothelium is the major indication for corneal transplantation and especially occurs in older people, an increase in the ageing population contributes to a growing need of healthy corneal endothelial cells suitable for transplantation.[6,7]

The two main cell-based alternatives to corneal endothelial transplantation that are currently explored are endothelial cell sheet transplantation and endothelial cell injection.

Endothelial cell sheet transplantation is still at a pre-clinical stage and a suitable cell carrier is required, either natural (denuded Descemet's membrane, decellularized corneas, human anterior lens capsules) or bioengineered, made of biocompatible materials.[8-22] Despite promising results of these newly-developed tissue engineered approaches, more *in vitro* and *in vivo* tests are needed, while issues such as legislation and reliability of a robust CEC culture protocol also need to be addressed. On the other hand, endothelial cell injection is already at

the clinical stage: cultured hCEC are injected into the anterior chamber together with a Rho-kinase (ROCK) inhibitor, which enhances cell proliferation.[23,24] The first clinical results from a group of 20 patients showed promising outcomes in terms of restored vision with excellent best corrected visual acuity (BCVA).[24]

Primary hCEC sources for research purposes are donor corneas which are not suitable for transplantation. While this does not solve the supply problem, cultured hCEC can be expanded *in vitro*, so that one donor cornea would provide the cells required to treat several patients. However, a cell culture protocol for hCEC should ideally follow the good manufacturing practice (GMP) guidelines for clinical application. GMP guidelines ensure that a manufactured product is safe for human use and provide a guidance in terms of manufacturing, testing and quality control. In the specific case of the hCEC culture protocol for clinical application, this means that all the products used should be GMP compliant and that the various steps of the protocol should be performed in controlled manufacturing facilities, under clearly defined and recorded processes and with a release system that minimize the risk to the quality and the safety of the product.[25]

This thesis describes the improvements on cell-based alternatives to standard corneal endothelial transplantation by bridging the gap between *in vitro* experiments and clinical models. The first part of this thesis focuses on the challenges in the elaboration of a robust and reproducible protocol for *in vitro* hCEC isolation and culture. The second part of the thesis is about endothelial cell sheet transplantation, from the expansion of both human and animal CEC on biocompatible carriers in order to evaluate the most suitable tissue-engineered substrates as corneal artificial transplants, to the *in vivo* application of such CEC-carrier constructs in animal models.

Optimization of the cell culture protocol for human corneal endothelial cells

It is known that hCEC isolated from younger donors have a higher *in vitro* proliferation rate than cells isolated from elderly donors, while the majority of donor corneas ineligible for transplantation derives from elderly donors.[26,27] As

we mainly worked with corneas from elderly donors, we first aimed at improving the success rate of establishing successful hCEC cultures for cells derived from those donor corneas. The strategy was to apply a "modified" dual-media approach similar to the study of Peh et al. on *in vitro* hCEC culture.[28] In our case, we applied the concept of growth factor-depleted medium to store the peeled Descemet's membrane-endothelial cell (DM-EC) complexes before subjecting cells to the cell isolation procedure. The rationale behind this concept was that the mechanical stress induced during the DM-EC preparation would have been a factor to reduce the population of viable hCEC,[29] given that non-viable and apoptotic cells are capable of negatively influencing their viable neighbors by the secretion of several factors;[30,31] therefore, the storage of the freshly peeled DM-EC sheet in a storage medium with no additional growth factors would increase the ratio of viable hCEC.

In our experimental set-up, the DM-EC sheets were isolated from paired donor corneas and exposed to two different experimental conditions: (1) cell isolation immediately after DM-EC peeling and (2) cell isolation after a period of up to 6 days of storage in a growth factor-depleted medium. Our results, described in **Chapter 3,** showed that cultures established from condition #1 reported a higher success rate compared to the cultures derived from condition #2. A positive stabilization effect was also reported in terms of cell proliferation, with cultures established from condition #1 reaching cell confluency faster than the cultures derived from condition #2. Taken together, these results indicate that targeting the non-viable hCEC population before cell isolation could be an important aspect in the establishment of a reproducible *in vitro* hCEC isolation and culture protocol. Moreover, this study opens up the possibility to successfully use endothelial grafts from elderly donors for cell isolation.
Our study was conducted on single donor corneas, an important aspect to evaluate in terms of clinical application of cultured hCEC. This approach enables tissue traceability and in turn reduces the risk of rejection upon hCEC transplantation. Tissue traceability and donor-to-donor variability are more challenging to evaluate in other hCEC culture protocols, where research-grade donor tissues are pooled together before cell isolation.[32]

The characteristics of the donor corneas used for our study, such as high donor age, low endothelial cell density, and long preservation time, were

disadvantageous for the establishment of primary hCEC cultures. Therefore, a better outcome for hCEC cultures established from younger donors is to be expected, as these corneas will have a higher cell density and proliferative capacity to start with.[26,33] First results about the application of the "modified" dual-media approach on younger donor corneas not suitable for transplantation (median age 53 years old, range 4-71 years old) showed that hCEC isolated following DM-EC incubation in M5-Endo medium for 48 hours before isolation were smaller and more homogenous than those harvested from the DM-EC incubation in M4-F99 medium. Cultured hCEC were then used for cell injection therapy for the treatment of corneal endothelial dysfunction in a rabbit model.[34]

Other research groups have outlined the importance and the feasibility to consider the research-grade corneas from elderly donors as suitable tissues for *in vitro* hCEC isolation and culture. Parekh et al. obtained hCEC cultures from old donor corneas in the presence of hyaluronic acid (HA) and Rho-kinase (ROCK) inhibitor Y-27632. Upon the evaluation of early attachment rates of hCEC on coated chamber slides and cell proliferation, they observed that isolated hCEC reached confluency within 10-15 days when cultured with HA + ROCK inhibitor.[35] Moreover, cultured hCEC showed a hexagonal morphology with very few cells showing polymorphism (less than 10%) and the amount of cells obtained at the end of confluency (about 2400 cells/mm^2) was considered as "transplantable" for penetrating keratoplasty (PK). Another study from the same research group reported that the use of a viscoelastic solution (Viscoat) could be beneficial in the passaging of hCEC isolated from old donors. After isolation, hCEC were seeded and left to attach after a topical application of chondroitin sulphate OVD (ophthalmic viscosurgical device). This treatment led to an accelerated attachment of passaged hCEC, without loss of cell morphology and marker expression.[36] Moreover, more proliferative cells were seen in the cultures with OVD compared to the cultures without OVD.

All the cell isolation protocols from elderly donors should be improved in a way to obtain hCEC cultures from single corneas. Otherwise, if several donor corneas are pulled together, it will be difficult to link the outcome of cell culture with donor characteristics, as stated by the researchers themselves, seen that also cell cultures established from younger donors could sometimes result in a failure.[36] Moreover, because Trypan blue staining is not able to distinguish

between apoptotic and dead cells,[37] an improvement on DM-EC sheets staining for cell viability is required to have a better picture of the overall cell population on the donor corneas, as well as to help biobankers and eventually surgeons in the evaluation of the quality of available donor tissue.

Adherence of the cell culture protocol to the GMP guidelines

To allow the translation from bench to clinic of *in vitro* cultured hCEC, a suitable culture protocol has to comply with regulatory directives. The good manufacturing practice (GMP) directives provide guidelines for manufacturing, testing and quality assessment of many products, including medical devices, in order to make sure that such products are suitable and safe for human use.[38] GMP directives are all based on defined core principles, but they may differ depending on the country or region in which they are applied.[38-42]
Cell-based alternatives to corneal transplantation which are considered to be advanced therapy medicinal products (ATMP), fall under the respective regulatory directives, to provide a safe product to patients. Starting from *in vitro* hCEC culture, the use of digestion enzymes (i.e. collagenase), fetal bovine serum (FBS), growth factors to be added in culture media, and surface coatings for cell culture may provide challenges to fully comply with the GMP process, as their efficacy depends on many variables such as source and batch-to-batch variations.[43] Nevertheless, safe and xeno-free alternatives to the above mentioned compounds are gradually being developed and becoming commercially available, for many cell isolation protocols from tissues as well as for *in vitro* hCEC culture and expansion.[18,44-49]

Within our process of the elaboration of a robust and reproducible hCEC culture protocol following GMP guidelines and suitable for clinical applications, we tested the effects of a GMP-grade collagenase, namely NB collagenase, on the establishment of hCEC cultures from elderly donors. As we showed in **Chapter 4**, the first difference we noticed between the GMP-grade collagenase and the research-grade collagenase that we used previously was the mechanism of action towards the DM-EC sheet, as the GMP-grade collagenase digestion resulted in small fragments of EC sheet, while the digestion mediated by the research-grade collagenase produced hCEC clusters.

The GMP-grade collagenase used in our study was previously used for human pancreatic islet isolation,[50] but its efficacy was not tested yet on hCEC isolation. Interestingly, our results showed a 4-times higher cellular yield after DM-EC digestion with the GMP-grade collagenase compared to the digestion with the research-grade isolation, despite a higher concentration needed for the efficacy of the GMP-grade collagenase. This might be related to the characteristic mechanism of action of the GMP-grade collagenase, as the presence of EC sheet fragments could facilitate hCEC expansion in the very early stages of the cell culture protocol and therefore establish primary cultures that reach cell confluency faster than cultures established from DM-EC sheet digested with research-grade collagenase and cells in the confluent monolayer showed good morphology.

No difference in time-to-reach cell confluency was observed after the first passage. These data are in contrast to the results described by Peh et al., in which they compared the efficacy of a GMP-grade collagenase blend (Liberase TH) and a research-grade collagenase (Collagenase Type 1).[18] In this case, the researchers did not find any differences in terms of cellular yield after tissue digestion. Nevertheless, the reason for the difference might reside in the fact that their study was conducted using research-grade corneas from younger donors (median age of 22 years old), while our study used elderly donors (median age of 73 years old).

However, despite the initial high cell density and hexagonal morphology at P0, at second passage cells isolated with GMP-grade collagenase in our study, were of large size and showed lack of contact inhibition. This may indicate that the initial cell isolation process may induce more cellular stress as compared to the research-grade collagenase, which upon passaging results in a decrease in proliferative capacity. Additional studies on the dependency of induced cellular stress on collagenase concentration may be required before incorporating this tested GMP-grade collagenase into culture protocols and Liberase TH may be a more suitable GMP-alternative.

Biomechanical properties and adhesion of porcine corneal endothelial cell – carrier constructs

Corneal endothelial cell sheet transplantation is a cell-based approach still at a pre-clinical stage, unlike endothelial cell injection. Endothelial cell sheet transplantation relies on the *in vitro* expansion of cultured hCEC on a bioengineered carrier, that can be of a natural or biocompatible nature, to provide a feasible and safe alternative to scarce human donor tissue. An ideal carrier should be transparent, not too thin, but flexible and biocompatible. Moreover, one should be able to determine the viability of the resulting cultured hCEC-carrier constructs as an additional quality control. All these requirements would finally lead to the possibility to "mimic" an original DMEK-graft, as the biomechanical properties should allow interaction with the recipient stroma.[51] On the other hand, the same properties should not interfere with the handling during the surgical procedure. Taken together, the compliance to these requirements would provide a clinical product that would constitute an alternative source for endothelial transplantation.

Our research focused on the feasibility of several biocompatible carriers, both bioengineered and natural, as potential alternatives to an endothelial graft for corneal endothelial transplantation. We first examined two types of substrates: GMP-produced porcine collagen scaffolds and human anterior lens capsules (HALC). Both types of carriers are already known as substrates for cell-based treatments for ocular reconstruction.[15,52] Cultured porcine corneal endothelial cells (pCEC) were used for these tests, for several reasons: pCEC are widely used in *in vitro* studies[53-56] and can be expanded more rapidly than cultured hCEC in order to guarantee a more efficient screening of the carrier material. As described in detail in **Chapter 5**, we showed that pCEC can be successfully cultured on collagen-based biocompatible carriers of different thickness (20 μm and 100 μm) and on HALC. Light microscopy analysis and expression of proliferation markers confirmed the ability of cultured pCEC to expand and proliferate on the abovementioned carriers. Carriers were then tested upon *in vitro* surgery in an experimental set-up composed of an artificial chamber and a human anterior remnant, namely a donor cornea without the endothelium. The pCEC-carrier constructs were tested according to some surgical parameters: rolling in BSS, staining with Trypan Blue, implantation in the artificial chamber, unfolding,

transparency and adherence to the posterior stroma. The pCEC-HALC constructs turned out to be the most similar to a DMEK graft, used as the reference model, while the biomechanical properties of the collagen-based carriers had an influence on their surgical behavior, in particular in terms of elasticity and tensile strength.

These results may suggest HALC to be a potential carrier for corneal endothelial cell sheet transplantation, as it has been shown that hCEC cultured on HALC retained their endothelial morphology and expressed typical markers ZO-1 and Na^+/K^+ - ATPase.[15] HALC is a transparent membrane that protects the lens from infections and is also able to shape the lens and its surface.[57,58] An adult HALC is composed of interacting networks of mainly laminin[59-62] and collagen type IV,[59,63,64] while other proteoglycans, such as collagen type XVIII, collagen type XV, perlecan and fibronectin, can be also found.[65,66] The benefits of HALC as cell carrier mostly come from its composition, that resembles that of the native DM: the core molecules self-establish a 3D matrix that gives strength, flexibility and signaling roles to the HALC,[67] although biomechanical tests show that these structural properties vary at different locations and with aging.[68] Nevertheless, using HALC does not solve the dependence from donor tissue, as one donor eye is still required for the preparation of each graft.
On the other hand, although the collagen-based carriers tested could potentially guarantee a limitless source of matrices for endothelial cell sheet transplantation, their biomechanical properties still have to be finely regulated in order to improve surgical handling and adhesion to the recipient stroma.

Verification of the suitability of carriers for human endothelial cells expansion and endothelial cell sheet transplantation

Following the preliminary results on the testing of different cell carriers described in **Chapter 5,** we cultured hCEC on the same typology of carriers, namely natural and bioengineered. Cultured hCEC were expanded on three different substrates: HALC, a collagen-based carrier of 20 μm (LK20), and denuded DM (dDM). We decided to include dDM because it is a carrier of natural origin and it resembles very much the DMEK graft. Moreover, dDM has been extensively characterized as a feasible carrier for hCEC, given that the DM

itself can provide the desired transparency and biomechanical support to cell expansion.[9,18] In **Chapter 6**, we showed that hCEC grown on the selected carriers retained their endothelial morphology. The expression of typical markers ZO-1 and Na^+/K^+ - ATPase was uniform and consistent in hCEC expanded on HALC and LK20, while the structured surface pattern of the dDM seemed to impair the expression of Na^+/K^+ - ATPase in hCEC cultured on this carrier. *In vitro* surgeries on the same experimental set-up explained in **Chapter 5** proved that all cell-carrier constructs could be implanted into the artificial anterior chamber using a DMEK technique. The hCEC-HALC and hCEC-dDM constructs best resembled the DMEK graft used as a reference, while the hCEC-LK20 constructs showed some issues related to the adherence to the posterior stroma.

Our results confirmed that the bioengineering of the collagen-based carrier LK20 has still room for improvement, although the values obtained for hCEC-LK20 constructs in unfolding and adherence represent an improvement if compared to those of the pCEC-LK20 showed in **Chapter 5**. On the other hand, natural membranes HALC and dDM showed advantages as substrates for endothelial hCEC sheet transplantation, given the similarity of their biomechanical properties with those of a DMEK graft. However, although natural carriers represent a high-quality benchmark for bioengineered carriers in terms of needed surgical handling and manipulation, their application in endothelial cell transplantation still relies on donor tissue and is influenced by donor-to-donor variability. The latter statement particularly applies for the dDM carriers derived from elderly donors as used in our study.

The preparation of both HALC and dDM for tissue engineering purposes requires an enzymatic treatment, i.e. a combination of trypsin and ethylenediaminetetraacetic acid (EDTA), in order to remove the cellular layers.[11,15,18] Our results showed that the enzymatic treatment works well for HALC, delivering a cell carrier with a relatively smooth surface and with no cells present after the treatment. On the other hand, the removal of the endothelial layer from the DM, that affects also the cell-extracellular matrix (ECM) interaction mediated by proteins like fibronectin,[69] resulted in a highly-structured surface. This could be explained by the fact that, in our study, dDM carriers were derived from elderly donor corneas. Given the age of the donors (>70 years),

the denuded DM surface was highly structured and not smooth. This pattern, together with a likely lack of secretion of the required proteins for cell adhesion with the membrane beneath, seemed to impact the morphology of the seeded hCEC, and resulted in an impaired quality of hCEC as demonstrated also by the diffuse expression of the Na^+/K^+ - ATPase marker.
The dDM might thus be a more suitable carrier, and also serve as a control for other cell-carrier constructs, if it would originate from younger donors resulting therefore in a less "imprinted" and smoother surface. However, these donors are not available at our eye bank and in most other European eye banks, and they are also not available from cataract surgery. While it would be possible on paper to purchase them from e.g. American eye banks, it would be hard to apply any obtained knowledge towards the development of tissue-engineered cell-carrier constructs, as the availability of such carriers would be even more restricted than for other types of natural carriers.
Nevertheless, after *in vivo* experiments conducted on rabbit eyes, a first-in-man clinical trial for tissue-engineered corneal grafts composed of denuded DM was recently given approval by Health Sciences Authority in Singapore (Clinical Trial Certificate: CTC1800013) for the treatment of FECD and bullous keratopathy.[18]

In vivo endothelial cell sheet transplantation in an animal model

Before introducing the cell-carrier constructs clinically, *in vivo* studies in animal models are required, in order to identify optimal conditions for graft adhesion and maintenance of endothelial cell function *in vivo*, as well as to determine possible adverse reaction of the host to the transplanted tissue and vice versa. Primates and rabbits have been the animal models mostly used for these purposes,[18,20,70-73] but studies can also be found with other animal models such as rats and cats.[9,12,74-77] At the same time, pigs have been used for implantation of fish-scale biocorneas[78] and represent an interesting alternative to the aforementioned models, because of the many similarities with the biology of the human eye.

After having explored some of the potential combination of cell expansion conditions and suitable, tissue-engineered material for endothelial cell sheet transplantation, we performed *in vivo* studies in Göttingen minipigs by

transplanting pCEC-HALC constructs using a Descemet stripping endothelial keratoplasty (DSEK)-like technique (**Chapter 7**). The first intraoperative challenges occurred when it was complicated to maintain the anterior chamber of the minipig eye, due to vitreous pressure. Moreover, descemetorhexis turned out to be very difficult to perform, as the Descemet membrane adhered strongly to the stroma, and therefore the endothelium instead had to be scraped off in several minipigs. This in turn led to the rapid formation of corneal edema, resulting in a difficult positioning of the pCEC-HALC constructs in some cases. After one month of follow up, the development of a central retrocorneal membrane was observed in all minipigs, mainly around the incisions. This strong wound healing reaction appears to be characteristic of the pig cornea, as a study in pig-to-monkey keratoplasty demonstrated an enhanced retrocorneal membrane in all monkeys due to the activation of stromal keratocytes in the pig graft.[79] Taken together, these data indicate the non-suitability of the minipig eye as a model for endothelial keratoplasty studies.

Histological examination showed a fibrotic response with associated angiogenesis caused by descemetorhexis. Interestingly, such fibrosis was milder when the endothelium was scraped, and in eyes without implantation of a pCEC-HALC construct, the scraped area included endothelial cells in number and morphology comparable to the native pig cornea. This suggests that pCEC are able to replicate *in vivo*, unlike primate CEC.[80]
As a consequence the information to be obtained on the pCEC-HALC constructs from these experiments was very limited due to the surgical difficulties and the strong wound-healing reaction. Therefore, the main conclusion from these experiments was that minipigs are not a suitable animal model for testing tissue-engineered cell-carrier constructs and for endothelial keratoplasty studies in general. Nevertheless, we could show that the cell-carrier constructs can be sufficiently stained for visualization, injected onto the anterior chamber and positioned against the posterior stroma.

Additional *in vivo* test with rabbits are currently underway. The rabbit model is well established for the study of many corneal pathologies, including bullous keratopathy.[75] Although some additional measures have to be taken into account before the surgical procedure, like a standard phacoemulsification to remove the crystalline lens to allow more space for surgical maneuvers,[18,81] rabbits are widely

accessible and the removal of the Descemet membrane is relatively easy. Although specific attention is required to the *in vivo* proliferative capacity of rabbit CEC and the need to completely remove the rabbit endothelium before transplantation to avoid overgrowth by rabbit CEC, this animal model is promising for the transplantation of tissue-engineered endothelial grafts, as long as the cultured CEC on the engineered graft are labelled (i.e. DiI labelling) to be distinguishable from the native rabbit endothelial layer.

Future perspectives

Although corneal transplantation will remain the mainstay of therapy for the treatment of corneal endothelial pathologies, the global shortage of donor tissues initiated the development of alternative techniques that lead either to a more efficient use of donor tissue or to a more independence from donor tissue.

For the cell-based approaches described in this thesis, there are some important points that need to be addressed before the translation into clinical application. One aspect regards the need of further research in the field of bioengineered carriers for endothelial cell sheet transplantation. Many candidates are now suitable for culturing hCEC and transplanting such cell sheets in animal models, however, biomechanical properties can still be improved to guarantee cell adherence to the substrate and pumping functions of the cells on the carrier. Moreover, the implementation of larger clinical trials to evaluate safety and application of cell-based approaches is needed, which is usually associated with high costs.

Other strategies than the ones described are being explored to reduce the dependency from donor tissue. The development of molecular biology techniques such as clustered regularly interspaced short palindromic repeat (CRISPR) endonucleases, opens new perspectives in gene therapy applied to FECD. It is known from literature that a majority of FECD patients have a trinucleotide repeat expansion in the TCF4 gene.[82] First *in vivo* experiments in mice have demonstrated that, using CRISPR-Cas technology, Cas proteins can be programmed to selectively bind to trinucleotide DNA sequences in cells carrying a FECD-similar genotype, inhibiting the related mRNA molecules and

therefore disrupting the pathological effects caused by them.[83] However, several risks associated with the CRISPR-Cas technology such as targeting efficiency, off-target effects and immunogenicity still represent a major setback towards clinical application.[84]

In the last years, 3D bioprinting is rapidly becoming a promising approach for corneal replacement. Main advantages of this techniques are the high degree of customization of the corneal implants, the defined control of shape and biomechanical properties and the possibility to adopt either single- and multi-layer corneal equivalents, according to the surgical needs.[85] A combination between gene therapy and 3D bioprinting applied to hCEC has been recently described, in which cultured hCEC were transfected with a plasmid to overexpress ribonuclease 5 (R5), a protein already known for promoting cell survival in many cell types[86] including cultured hCEC.[87] R5 overexpression was a steady requirement for the following bioprinting steps, as R5-hCEC were suspended in gelatin-based bioink, and then printed on a lyophilized bovine amniotic membrane. This led to a 3D-printed construct made by 7 layers and with a thickness of 700 μm and a cell density of more than 3000 cells/mm^2. Upon transplantation in a rabbit model, the 3D graft started to restore the clarity of rabbit corneas, while the expression of typical corneal endothelial markers was more prominent on the 3D grafts than on the controls at 4 weeks after transplantation.[88]

Despite the latest progresses, these strategies are still at an experimental stage and therefore have to fulfill several requirements before being considered as clinical-grade approaches for the treatment of corneal pathologies in the near future. Gene therapy requires *in vivo* testing first on animal models and then in humans, including strict safety requirements, while 3D corneal bioprinting is hampered for wider applications by its high costs.

Conclusive remarks

The studies presented in this thesis show that corneal endothelial cell sheet transplantation has taken huge steps towards the clinical application and could represents a promising alternative to corneal transplantation. Future research

should be directed to a better definition of the hCEC culture condition, from the improvement of the graft storage by the development of bioreactors that simulate *in vivo*-like culture conditions to the development of GMP-compliant alternatives to the components currently used in *in vitro* hCEC culture, to a better identification of hCEC used for clinical application by means of fluorescence-activated cell-sorting (FACS) analysis,[24] cytokine level analysis,[89] and transcriptomic analysis.[90] In parallel, more effort is needed in the search of the "ideal" tissue-engineered carrier for transplantation, that has to guarantee a firm support for cultured hCEC, with biomechanical and biocompatible properties similar to the native membranes. The optimization of these two combined research lines will increase our possibility to translate the corneal endothelial cell sheet transplantation from the bench to the clinic, and may contribute to overcome the global shortage of donor tissue and to implement in the future a patient-specific treatment for millions of people affected by corneal endothelial pathologies.

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8

CHAPTER 9

Nederlandse Samenvatting

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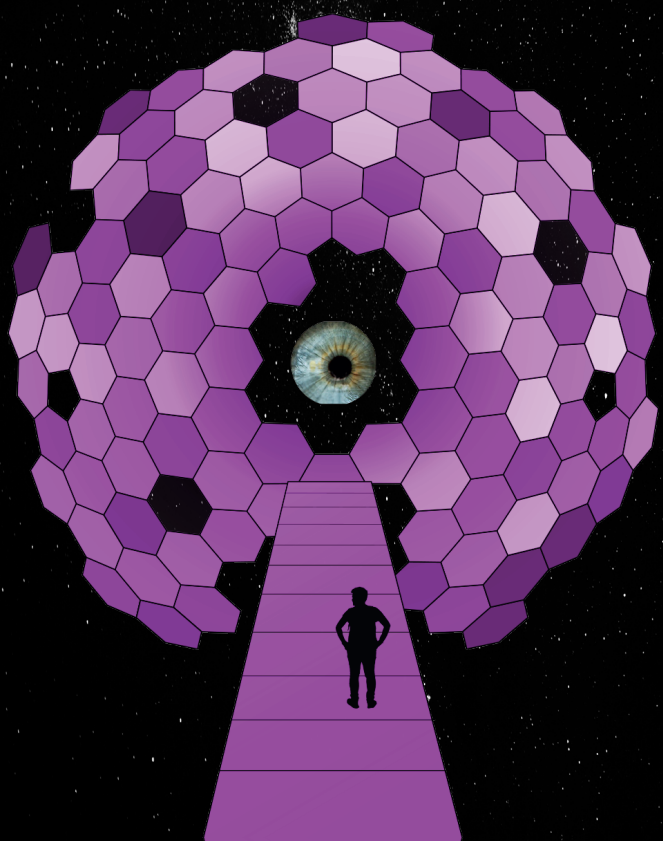

NEDERLANDSE SAMENVATTING

De ontwikkeling van de Descemet membraan endotheel keratoplastiek (DMEK) vormt een mijlpaal in de geschiedenis van de hoornvliestransplantatie en is tot op heden de meest selectieve chirurgische techniek om cornea endotheelafwijkingen te behandelen, omdat het de vervanging van het beschadigde cornea endotheel door gezonde donorweefsel toestaat.[1] Helaas is de DMEK strikt afhankelijk van de beschikbaarheid van donorweefsel.

Om het wereldwijde tekort aan donorweefsel te overwinnen, zijn nieuwe benaderingen nodig, samen met chirurgische benaderingen die gericht zijn op het optimaliseren van het gebruik van donorendotheel voor transplantaties. De op cellen gebaseerde strategieën zijn gebaseerd op de *in vitro* expansie en kweek van menselijke cornea endotheel cellen (humane cornea endotheleel cellen, hCEC), die zich achter op het hoornvlies bevinden, waar ze een enkele cellaag vormen, en verantwoordelijk zijn voor het behoud van de hoornvliesdikte en transparantie.[2] Aangezien hCEC slecht delen,[3-5] zal dood of dysfunctie van endotheelcellen uiteindelijk leiden tot een afname of verlies van cornea transparantie. Omdat verlies van cornea endotheelcellen de belangrijkste indicatie is voor hoorvliestransplantaties en vooral nodig is bij oudere mensen, draagt een toename van de vergrijzende bevolking bij aan een groeiende behoefte aan gezonde cornea endotheel cellen die geschikt zijn voor transplantatie.[6,7]

De twee belangrijkste op cellen gebaseerde alternatieven voor cornea endotheel transplantatie die momenteel worden onderzocht zijn endotheel celbladtransplantatie en endotheel celinjectie.
Endotheel celbladtransplantatie bevindt zich nog in een preklinisch stadium en er is een geschikte celdrager voor nodig, ofwel een natuurlijklijke (gedecellariseerde Descemet membraan, gedecellariseerde corneas, menselijke anterieure lenskapsel), ofwel een drager gemaakt van biocompatibele materialen.[8-22] Ondanks veelbelovende resultaten van deze nieuwe benaderingen zijn er meer *in vitro* en *in vivo* tests nodig, terwijl kwesties als wetgeving en betrouwbaarheid van de kweekprotocollen ook moeten worden aangepakt. Aan de andere kant bevindt de injectie van losse endotheelcellen zich al in het klinische stadium: gekweekte hCEC worden samen met een Rho-kinase

(ROCK)-inhibitor (wat de celproliferatie bevordert) in de voorste kamer ingespoten.[23,24] De eerste klinische resultaten van een groep van 20 patiënten toonden veelbelovende resultaten in termen van herstelde visus met uitstekende best gecorrigeerde visuele scherpte (best corrected visual acuity, BCVA).[24]

De primaire bron voor hCECs vormed donorcorneas die niet geschikt zijn voor transplantatie. Hoewel dit het leveringsprobleem niet oplost, kunnen gekweekte hCEC *in vitro* worden vermeerderd, zodat één donorhoornvlies de cellen zou kunnen leveren voor meerdere patiënten.

Dit proefschrift beschrijft de verbeteringen van celkweken voor cornea endotheelcel transplantatie om de kloof tussen *in vitro* experimenten en klinische modellen te overbruggen. Het eerste deel van dit proefschrift richt zich op de uitdagingen bij het opstellen van een robuust en reproduceerbaar protocol voor *in vitro* hCEC-isolatie en -celkweek, dat idealiter zou moeten voldoen aan de richtlijnen voor "good manifacturing practice", GMP, voor naleving van regelgeving. Het tweede deel van de thesis gaat over endotheelcel transplantatie, van de vermeerdering van zowel menselijke als dierlijke CEC op biocompatibele dragers om de meest geschikte substraten voor corneacel transplantaties te evalueren tot de *in vivo* toepassing van dergelijke CEC-dragerconstructies in diermodellen.

Optimalisatie van het celkweekprotocol voor menselijke cornea endotheel cellen

Het is bekend dat hCECs geïsoleerd van jonge donoren een hogere *in vitro* proliferatie tonen dan cellen geïsoleerd van oude donoren, terwijl het merendeel van de donorcorneas die niet in aanmerking komen voor transplantatie juist afkomstig zijn van oude donoren.[25,26] Omdat we daarom vooral met corneas van oude donoren werken, hebben we ons eerst gericht op het verbeteren van het succespercentage van het opzetten van succesvolle hCEC-culturen voor cellen die afkomstig zijn van die donorcorneas. De strategie was om een "gewijzigde" dual-media benadering toe te passen, vergelijkbaar met de studie van Peh et al. betreffende *in vitro* hCEC kweken.[27] In ons geval hebben we het concept van groeifactor-uitgeput medium toegepast om de Descemet membraan-endotheel

celcombinaties (DM-EC) op te slaan voordat we cellen onderwierpen aan de celisolatieprocedure. Wij hadden als veronderstelling dat de mechanische stress, die tijdens de voorbereiding van de DM-EC optrad, het aantal levensvatbare hCECs zou verminderen:[28] niet-levensvatbare en apoptotische cellen kunnen hun gezonde buren negatief te beïnvloeden door de afscheiding van verschillende factoren.[29,30] Indien het vers verkregen DM-EC complex eerst bewaard wordt in een medium zonder extra groeifactoren, dan zou het percentage levensvatbare hCEC derhalve moeten verbeteren.

In onze experimentele opzet gebruikten we de twee DM-EC-bladen afkomstig van de twee ogen van één donor, en stelden ze bloot aan twee verschillende experimentele omstandigheden: (1) celisolatie onmiddellijk na het verkrijgen van de DM-EC en (2) celisolatie na een periode van maximaal 6 dagen opslag in een medium met een groeifactor. Onze resultaten, beschreven in **Hoofdstuk 3**, toonden aan dat culturen die zijn ontstaan onder voorwaarde #1 een hoger succespercentage meldden in vergelijking met de culturen onder voorwaarde #2. Er was ook een beter effect op celproliferatie, waarbij kweken onder voorwaarde #1 sneller confluentie bereikten dan de kweken onder voorwaarde #2. Samen wijzen deze resultaten erop dat het kwijtraken van de niet-levensvatbare hCEC-populatie vóór celisolatie een belangrijk aspect kan zijn bij de totstandbrenging van een reproduceerbaar *in vitro* hCEC-isolatie- en kweekprotocol. Bovendien biedt dit onderzoek de mogelijkheid om corneas van oudere donoren succesvol te gebruiken voor celisolatie.

Ons onderzoek werd uitgevoerd op enkelvoudige donorcorneas, een belangrijk aspect bij klinische toepassing van gekweekte hCEC. Deze aanpak maakt het naar de donor traceren van weefsel mogelijk en vermindert op zijn beurt het risico van afstoting bij hCEC-transplantatie. De traceerbaarheid van weefsel en de variabiliteit tussen donor en donor zijn lastiger te evalueren wanneer donorweefsels in kweek worden samengevoegd.[31]

De kenmerken van de donorcorneas die voor ons onderzoek werden gebruikt, zoals een hoge leeftijd van de donor, een lage dichtheid van endotheelcellen en een lange bewaartijd, waren nadelig voor de primaire hCEC-kweken. We kunnen een betere uitkomst verwachten bij gebruik van jongere donoren, omdat deze cornea's starten met een hogere celdichtheid en een grotere

productiecapaciteit.[25,32] Ook andere onderzoeksgroepen hebben getest of de cornea van oude donoren geschikt is voor *in vitro* hCEC-isolatie en -kweek. Parekh et al. verkregen hCEC-culturen uit oude donorcorneas in aanwezigheid van hyaluronzuur (hyaluronic acid, HA) en de Rho-kinase (ROCK) inhibitor Y-27632: wanneer geïsoleerde hCEC samen met HA en Rock inhibitor werden gekweekt, bereikten ze binnen 10-15 dagen confluentie.[33] Bovendien toonden de gekweekte hCEC een zeshoekige morfologie met zeer weinig cellen met polymorfisme (minder dan 10%); de hoeveelheid cellen (ongeveer 2400 cellen/mm^2) kon worden beschouwd als "transplanteerbaar". Een ander onderzoek van dezelfde onderzoeksgroep rapporteerde dat het gebruik van een viscoelastisch middel (Viscoat) nuttig zou kunnen zijn bij oude donoren. Na isolatie werden hCEC gezaaid met toepassing van Viscoat. Deze behandeling leidde tot een versnelde hechting van hCEC, zonder verlies van celmorfologie en markerexpressie.[34] Bovendien werden er in de culturen met Viscoat meer levende cellen waargenomen in vergelijking met de culturen zonder Viscoat.

De protocollen voor celisolatie van oude donoren moeten nog verder worden verbeterd om genoeg hCECs te verkrijgen. Indien het nodig blijft om cellen uit meerdere donorcorneas's bijeen te voegen, zal het moeilijk zijn om de uitkomst van de celkweek te koppelen aan de kenmerken van donoren, omdat ook celculturen die zijn ontstaan uit jonge donoren soms tot een mislukking kunnen leiden.[34] Omdat Trypan Blue Staining geen onderscheid maakt tussen apoptotische en dode cellen,[35] is een andere benadering nodig om de levensvatbaarheid van cellen te bepalen en daarmeeeen beter beeld te krijgen van de totale celpopulatie op de donorcorneas, en om biobankers en uiteindelijk chirurgen te helpen bij de evaluatie van de kwaliteit van het beschikbare donorweefsel.

Naleving van het protocol voor celcultuur van de GMP richtlijnen

Om de vertaling van de *in vitro* gekweekte hCEC van de bank naar de kliniek mogelijk te maken, moet een geschikt kweekprotocol voldoen aan wettelijke richtlijnen. GMP richtlijnen bestaan voor de productie, het testen en de kwaliteitsbeoordeling van veel producten, inclusief medische hulpmiddelen, om ervoor te zorgen dat dergelijke producten geschikt en veilig zijn voor menselijk

gebruik.[36] GMP richtlijnen zijn allemaal gebaseerd op gedefinieerde kernbeginselen, maar kunnen verschillen afhankelijk van het land of de regio waar ze worden toegepast.[36-40]
Celgebaseerde alternatieven voor cornea transplantatie worden beschouwd als advanced therapy medicinal products (ATMP) en vallen onder de hiervoor geldende specifieke wettelijke richtlijnen. Bij de *in vitro* hCEC-kweek wordt gebruik gemaakt van enzymen (d.w.z. collagenase), foetaal runderserum (fetal bovine serum, FBS), groeifactoren en oppervlaktecoatings; dit alles kan uitdagingen opleveren om volledig aan het GMP proces te voldoen, omdat de werkzaamheid van deze factoren afhankelijk is van veel variabelen zoals batch-to-batch verschillen.[41] Niettemin worden er geleidelijk veilige en xeno-vrije alternatieven voor de bovengenoemde stoffen ontwikkeld en commercieel beschikbaar gemaakt voor veel celisolatieprotocollen en voor *in vitro* hCEC-kweek en -expansie.[18,42-47]

Om te komen tot een robuust en reproduceerbaar hCEC-kweekprotocol volgens GMP richtlijnen voor klinische toepassingen, hebben we de effecten getest van een GMP-grade collagenase bij hCEC-kweken van oudere donoren. Zoals we in **Hoofdstuk 4** hebben aangetoond, zagen we een verschil tussen de collagenase van GMP-kwaliteit en de collagenase van onderzoekskwaliteit die we eerder gebruikten: de collagenase van GMP-kwaliteit leidde tot fragmentatie van het DM-EC-blad, terwijl de digestie door de onderzoekskwaliteit collagenase hCEC-clusters produceerde.

De collagenase van GMP-kwaliteit die wij testten werd reeds gebruikt voor de isolatie van menselijke alvleesklieреilandjes;[48] de werkzaamheid ervan was nog niet eerder getest voor isolatie van hCECs. Interessant genoeg toonden onze resultaten een 4 maal hogere cellulaire opbrengst bij gebruik van de collagenase van GMP-kwaliteit in vergelijking met het gebruik van de onderzoekskwaliteit collagenase, ondanks dat er een hogere concentratie nodig wasvan de laatste. Na de eerste passage werd geen verschil in tijd-tot-confluentie waargenomen. Deze gegevens staan in contrast met de resultaten die worden beschreven door Peh et al., waarin eveneens de werkzaamheid van een collagenase-mengsel van GMP-kwaliteit (Liberase TH) en een collagenase van onderzoekskwaliteit (Collagenase Type 1) werdt vergeleken.[18] In dit geval vonden de onderzoekers geen verschillen in termen van cellulaire opbrengst na digestie. Niettemin kan de reden voor het

verschil liggen in het feit dat hun onderzoek werd uitgevoerd met corneas van onderzoekskwaliteit van jonge donoren (mediaan van 22 jaar), terwijl ons onderzoek oude donoren (mediaan van 73 jaar) gebruikte.

Ondanks de aanvankelijke hoge celdichtheid en zeshoekige morfologie bij P0 waren de cellen in onze studie, die in tweede passage met GMP-grade collagenase werden geïsoleerd, van grote omvang en toonden zij gebrek aan contactremming. Dit kan erop wijzen dat het initiële celisolatieproces meer celspanning kan veroorzaken in vergelijking met de collagenase van onderzoekskwaliteit, die bij celpassages resulteert in een afname van de productieve capaciteit. Aanvullende studies over de afhankelijkheid van geïnduceerde cellulaire stress bij collagenase-concentratie kunnen nodig zijn voordat de geteste collagenase van GMP-kwaliteit in kweekprotocollen kan worden opgenomen.

Biomechanische eigenschappen en adhesie van de endotheelcel van varkenscornea – dragerconstructies

Cornea endotheel celtransplantatie is een op cellen gebaseerde benadering die nog in een preklinisch stadium verkeert, in tegenstelling tot endotheel celinjectie. Endotheel celtransplantatie is afhankelijk van de *in vitro*-uitbreiding van gekweekte hCEC op een drager, die van natuurlijke of biocompatibele aard kan zijn, om een haalbaar en veilig alternatief te bieden voor schaars menselijk donorweefsel. Een ideale drager moet transparant zijn, niet te dun, maar flexibel en biocompatibel. Bovendien moet men in staat zijn de levensvatbaarheid van de daaruit voortvloeiende gekweekte hCEC-dragerconstructies te bepalen. Al deze eisen zullen uiteindelijk leiden tot de mogelijkheid om een originele DMEK-graft te "minimaliseren", omdat de biomechanische eigenschappen interactie met het ontvangende stroma zouden moeten toestaan.[49] Aan de andere kant mogen dezelfde eigenschappen de behandeling tijdens de chirurgische ingreep niet verstoren. Samen zou de naleving van deze eisen een klinisch product opleveren dat een alternatieve bron zou vormen voor endotheel transplantatie.

Ons onderzoek richtte zich op de haalbaarheid van verschillende biocompatibele dragers, zowel biotechnische als natuurlijke, als potentiële alternatieven voor een endotheel transplantatie zoals een DMEK. We hebben eerst twee soorten substraten onderzocht: door GMP geproduceerde varkenscollageen dragers en menselijke lenskapsels (human anterior lens capsule, HALC). Beide typen dragers worden als substraten genoemd voor celgebaseerde behandelingen voor oculaire reconstructie.[15,50] Voor deze tests werden gekweekte cornea endotheel cellen van varkens (porcine corneal endothelial cells, pCEC) gebruikt, om de volgende redenen: pCECs worden veel gebruikt in *in vitro*-studies[51-54] en kunnen sneller vermeerderen dan gekweekte hCECs om een efficiëntere screening van het draagmateriaal te garanderen. Zoals in **Hoofdstuk 5** beschreven, hebben we aangetoond dat pCECs succesvol kunnen worden gekweekt op collageen gebaseerde biocompatibele dragers van verschillende dikte (20 μm en 100 μm) en op HALC. Microscopisch onderzoek en analyse van de expressie van proliferatiemerkers bevestigden dat gekweekte pCEC het vermogen hebben om zich uit te breiden en te verspreiden op de bovengenoemde dragers. De dragers werden vervolgens chirurgisch getest in een experimentele opstelling bestaande uit een kunstmatige voorste oogkamer met daarin een menselijk donorhoornvlies zonder endotheel. De pCEC-dragerconstructies werden getest volgens enkele chirurgische parameters: het kunnen oprollen in balanced salt solution, kleuring met Trypan Blue, implantatie in de kunstmatige kamer, ontvouwen, transparantie en de hechting aan het posterieure stroma. De pCEC-HALC-constructies bleken het meest te lijken op een DMEK-graft, gebruikt als referentiemodel, terwijl de biomechanische eigenschappen van de collageen-gebaseerde dragers een invloed hadden op hun chirurgische gedrag, vooral in termen van elasticiteit en treksterkte.

Deze resultaten suggereren dat HALC een potentiële drager is voor cornea endotheel celtransplantatie, omdat is aangetoond dat op HALC gekweekte hCECs hun endotheel morfologie hebben behouden en de typische endotheelmarkers ZO-1 en Na^+/K^+ - ATPase tot uitdrukking brengen.[15] HALC is een transparant membraan dat de lens beschermt tegen zijn omgeving en de lensvorm bepaalt.[55,56] Een volwassen HALC bestaat uit netwerken van voornamelijk laminine-[57-60] en collageen type IV,[57,61,62] terwijl andere proteoglycanen, zoals collageen type XVIII, collageen type XV, perlecaan en

fibronectine, ook worden aangetroffen.[63,64] De voordelen van HALC als celdrager berusten op de samenstelling, die lijkt op die van de oorspronkelijke DM: de kernmoleculen vormen een 3D-matrix die kracht, flexibiliteit en signaleringsfucnties aan het HALC geeft,[65] hoewel biomechanische testen aantonen dat deze structurele eigenschappen op verschillende plekken en met veroudering verschillen.[66] Helaas lost gebruik van HALC de afhankelijkheid van donorweefsel niet op, omdat er nog steeds één donoroog nodig is voor de voorbereiding van elke transplantatie.
Hoewel de geteste collageen-gebaseerde dragers potentieel een onbeperkte bron van matrices voor endotheel celbladtransplantatie kunnen garanderen, moeten hun biomechanische eigenschappen nog verder worden aangepast om de chirurgische behandeling en hechting aan het ontvangende stroma te verbeteren.

Verificatie van de geschiktheid van dragers voor de expansie en transplantatie van menselijke endotheel cellen

Na de voorlopige resultaten over het testen van verschillende celdragers (**Hoofdstuk 5**), hebben we hCEC op deze dragers gekweekt. De gekweekte hCEC werden uitgezaaid op drie verschillende substraten: op de HALC, op LK20, een op collageen-gebaseerde drager van 20 μm dikte, en op celvrij gemaakt DM (denuded DM, dDM). We besloten om dDM te gebruiken omdat het een drager is van natuurlijk materiaal die zeer veel lijkt op de DMEK-graft. Bovendien is dDM uitgebreid gekarakteriseerd als een haalbare drager voor hCEC, gezien de DM zelf de gewenste transparantie en biomechanische ondersteuning biedt voor celvermeerdering.[9,18] In **Hoofdstuk 6** hebben we laten zien dat hCECs die op de geselecteerde dragers zijn gekweekt hun endotheel morfologie hebben behouden. HCEC die groeiden op HALC en LK20 toonden een uniforme en consistente expressie van de typische endotheelmarkers ZO-1 en Na^+/K^+ - ATPase, terwijl het gestructureerde oppervlaktepatroon van de dDM de expressie van Na^+/K^+ - ATPase in de hCEC leek te schaden. *In vitro* operaties toonden aan dat alle celdragerconstructies met behulp van een DMEK techniek in de kunstmatige voorste oogkamer konden worden geïnplanteerd. De hCEC-HALC en hCEC-dDM leken in hun biomechanische gedrag het meest op de DMEK-graft die als referentie gebruikt werd, terwijl de hCEC-LK20-

constructies een aantal problemen lieten zien bij de adhesie aan het posterieure stroma.

Onze resultaten bevestigen dat er ruimte is voor verbetering van de eigenschappen van de collageen-gebaseerde drager LK20, hoewel er duidelijk verbetering was bij het gebruik van humane CECs in vergelijking met die van de varkens CEC-LK20 in **Hoofdstuk 5**. Hoewel natuurlijke dragers goed werkten, is hun toepassing bij endotheel celtransplantatie nog steeds afhankelijk van donorweefsel en kan deze worden beïnvloed door de variabiliteit tussen donoren. Dit geldt vooral voor de dDM-dragers afkomstig van oudere donoren zoals die in ons onderzoek zijn gebruikt.

Voor de bereiding van zowel HALC als dDM voor weefselengineering is een enzymatische behandeling nodig, d.w.z. een combinatie van trypsine en ethylenediaminetetraacetic acid (EDTA), om cellen te verwijderen.[11,15,18] Onze resultaten toonden aan dat de enzymatische behandeling goed werkt voor HALC, hetgeen resulteert in een celdrager met een relatief glad oppervlak zonder cellen. De DM beinvloedt ook de cel-extracellulaire matrix (extracellular matrix, ECM) interactie beïnvloedt, die wordt gemedieerd door eiwitten zoals fibronectine.[67] Verwijdering van de endotheelcellaag van de dDM resulteerde in een zeer gestructureerd oppervlak. Dit kan worden verklaard door het feit dat in ons onderzoek dDM-dragers zijn verkregen van oude donorcorneas. Door de leeftijd van de donoren (>70 jaar) was het dDM-oppervlakte zeer gestructureerd en niet glad. Dit patroon, samen met een waarschijnlijk gebrek aan afscheiding van de eiwitten die vereist zijn voor hechting van de cellen aan de onderliggende membraan, leek de morfologie van de gezaaide hCEC te beïnvloeden, en resulteerde in een verminderde kwaliteit van de hCEC, zoals ook blijkt uit de diffuse expressie van de Na^+/K^+ - ATPase marker.
De dDM zou dus een geschiktere drager kunnen zijn, en ook als controle voor andere celdragerconstructies kunnen dienen, als deze afkomstig zou zijn van jonge donoren, wat dus zou resulteren in een minder "druk" en gladder oppervlak. Deze donoren zijn echter niet beschikbaar bij onze oogbank en bij de meeste andere Europese oogbanken. Hoewel het op papier mogelijk zou zijn ze aan te schaffen bij bijvoorbeeld Amerikaanse oogbanken, zou het moeilijk zijn om de verkregen kennis toe te passen op de ontwikkeling van weefselgebonden celdragerconstructies, omdat de beschikbaarheid van

dergelijke dragers nog beperkter zou zijn dan voor andere soorten natuurlijke dragers.
Niettemin werd na *in vivo* experimenten met konijnenogen onlangs een eerste klinische proef voor celdrager cornea transplantaties, bestaande uit denuded DM, goedgekeurd door de Health Sciences Authority in Singapore (Clinical Trial Certificate: CTC1800013) voor de behandeling van FECD en bulleuze keratopathie.[18]

In vivo endotheel celdragertransplantatie in een diermodel

Voordat de celdragerconstructies klinisch worden geïntroduceerd, zijn studies in diermodellen vereist om de optimale omstandigheden voor hechting van de celdrager te bepalen en om de endotheelcelfunctie te evalueren. Verder dient onderzoek plaats te vinden naar afstotingsreacties. Primaten en konijnen worden vaak als diermodel gebruikt,[18,20,68-73] maar er zijn ook studies te vinden met andere diermodellen zoals ratten en katten.[9,12,74,75] Varkens zijn gebruikt voor de implantatie van visschub biocorneas[76] en vormen een interessant alternatief voor de bovengenoemde modellen vanwege de vele overeenkomsten met de biologie van het menselijk oog.

Nadat we eerst de optimalisering van celvermeerdering en het gebruik van een celdrager hebben onderzocht, hebben we *in vivo* studies uitgevoerd in Göttingen minipigs. We transplanteerden pCEC-HALC-constructies met behulp van een Descemet-stripping endotheel keratoplastiek (DSEK)-achtige techniek (**Hoofdstuk 7**). De eerste intraoperatieve uitdagingen traden op toen het ingewikkeld bleek om de voorste kamer van het minipig oog te handhaven, als gevolg van druk vanuit het glasvocht. Bovendien bleek het heel moeilijk om descemetorhexis uit te voeren, omdat het Descemet membraan niet los liet van het stroma; als alternatief moest het endotheel worden afgeschraapt. Dit leidde tot de snelle vorming van cornea oedeem, wat in sommige gevallen tot een moeilijke positionering van de pCEC-HALC-constructies leidde. Na een maand werd de ontwikkeling van een centraal retrocorneaal membraan waargenomen in alle minipigs, voornamelijk rond de incisies.
Deze krachtige reactie op wondgenezing lijkt kenmerkend voor de varkenscornea, omdat uit een onderzoek bij hoornvliestranplantatie van varkens

naar apen blijkt dat alle apen een retrocorneale membraan ontwikkelden door activering van stromale keratocyten in het varkensweefsel.[77] Deze gegevens leiden tot de conclusie dat het minipig oog niet geschikt is als model voor endotheel transplantatie studies.

Histologisch onderzoek toonde een fibrotische reactie aan met de bijbehorende bloedvatvorming veroorzaakt door de descemetorhexis. Interessant genoeg was de fibrose milder toen het endotheel werd afgeschraapt; in de ogen zonder implantatie van een pCEC-HALC-constructie bevatte het eerder afgeschraapte gebied toch endotheel cellen, die in aantal en morfologie vergelijkbaar waren met de oorspronkelijke varkenscornea. Dit duidt erop dat pCECs in de minipig in staat zijn om *in vivo* te repliceren, in tegenstelling tot primaten CEC.[78]
Als gevolg van de chirurgische problemen en de sterke reactie op wondgenezing was de informatie die we over de pCEC-HALC-constructies uit deze experimenten verkregen zeer beperkt. We hebben wel kunnen laten zien dat de celdragerconstructies voldoende aangekleurd konden worden voor visualisatie, in de voorste kamer konden worden geïnjecteerd en tegen het achterste stroma aan konden worden geplaatst.

Er is momenteel een extra *in vivo*-test met konijnen gaande. Het konijnenmodel is goed opgezet voor de studie van vele cornea afwijkingen, waaronder bulleuze keratopathie.[73] Hoewel er vóór de chirurgische ingreep rekening moet worden gehouden met enkele aanvullende maatregelen, zoals een standaard phacoemulsificatie om de kristallijne lens te verwijderen om meer ruimte te bieden voor chirurgische manoeuvres,[18,79] zijn konijnen breed beschikbaar en is het verwijderen van de Descemet membraan relatief eenvoudig. Vanwege de *in vivo*-proliferatieve capaciteit van konijnen CECs is het noodzakelijk om het konijnen cornea endotheel vóór transplantatie volledig te verwijderen om overgroei door konijnen CECs te voorkomen. Dit model lijkt veelbelovend voor het testen van weefselendotheel transplantaties, zolang de gekweekte CEC op de engineered graft gelabeld zijn (d.w.z. door DiI-etikettering) zodat ze te onderscheiden zijn van de eigen konijnenendotheel laag.

Toekomstperspectief

Hoewel hoornvliestransplantatie de gekozen therapie zal blijven voor de behandeling van cornea endotheel aandoeningen, heeft het wereldwijde tekort aan donorweefsel geleid tot de ontwikkeling van alternatieve technieken om te komen tot een efficiënter gebruik of een grotere onafhankelijkheid van donorweefsel.

Voor de cellulaire benaderingen die in dit proefschrift worden beschreven, zijn er nog enkele belangrijke punten die moeten worden aangepakt voordat de vertaling naar klinische toepassing kan plaatsvinden. Dit betreft oa. verder onderzoek naar dragers voor endotheel celtransplantatie. Er zijn meerdere dragers die reeds geschikt zijn om hCECs op te kweken en ze in in diermodellen in het oog te implanteren, maar de biomechanische eigenschappen kunnen nog verder worden verbeterd om de celhechting aan het substraat en de pompfuncties van de cellen op de drager te garanderen. Bovendien zijn klinische trials noodzakelijk om de veiligheid te evalueren, hergeen gewoonlijk gepaard gaat met hoge kosten.

Andere strategieën dan die hier beschreven zijn worden onderzocht om de afhankelijkheid van donorweefsel te verminderen. De ontwikkeling van moleculair biologische technieken zoals clustered regularly interspaced short palindromic repeat (CRISPR) endonucleases opent nieuwe perspectieven in gentherapie, die kan worden toegepast op Fuchs endotheeldystrofie (Fuchs endothelial corneal dystrophy, FECD). Uit de literatuur blijkt dat de meeste FECD-patiënten meerdere copiëen hebben van een trinucleotide in het TCF4-gen.[80] De eerste *in vivo* experimenten bij muizen hebben aangetoond dat Cas-eiwitten, met behulp van CRISPR-Cas-technologie, kunnen worden geprogrammeerd om selectief te binden aan deze trinucleotide DNA-volgorden in cellen met een FECD-vergelijkbaar genotype, waardoor de betrokken mRNA-moleculen worden geremd evenals hun pathologische werking.[81]

De afgelopen jaren wordt 3D-bioprinting een optie bij cornea vervanging. De belangrijkste voordelen van deze techniek zijn de hoge mate van aanpasbaarheid van het implantaat, de controle over de vorm en biomechanische eigenschappen en de mogelijkheid om één- en meerlaagse cornea equivalenten te gebruiken, afhankelijk van de chirurgische behoeften.[82] Een combinatie van gentherapie en

3D-bioprinten is onlangs beschreven, waarin gdmv. een plasmide extra ribonuclease 5 (R5), een eiwit dat al bekend staat om het bevorderen van celoverleving,[83] in gekweekte hCEC tot expressie werd gebracht.[84] R5-overexpressie was nodig voor de volgende stappen van het bioprinten, waarbij de R5-hCEC in een bioinkt op gelatinebasis werden afgedrukt op een gelyofiliseerd amnionmembraan van runderen. Dit leidde tot een 3D-geprinte constructie van zeven cellagen, met een dikte van 700 μm en een celdichtheid van meer dan 3000 cellen/mm^2. Dit transplantaat was in staat om in een konijnenmodel de helderheid van konijnencorneas te herstellen, terwijl er een betere expressie van typische cornea endotheel markers was op de 3D-grafts dan op de controles, 4 weken na transplantatie.[85]

Ondanks de vooruitgang bevinden deze strategieën zich nog in een experimenteel stadium en moeten ze nog aan allerlei eisen gaan voldoen voordat ze kunnen worden toegepast bij de behandeling van cornea aandoeningen. Gentherapie vereist *in vivo* testen, eerst op diermodellen en vervolgens bij mensen, inclusief strikte veiligheidseisen, terwijl de hoge kosten bij 3D cornea bioprinting belemmerend werken.

Conclusies

De studies die in dit proefschrift worden gepresenteerd tonen aan dat cornea endotheel celtransplantatie enorme stappen heeft gezet in de richting van de klinische toepassing en een veelbelovend alternatief kan vormen voor traditionele cornea transplantatie. Toekomstig onderzoek moet worden gericht op een betere definitie van de hCEC-kweekcondities, van de verbetering van de opslag van het donorweefsel door de ontwikkeling van kweekkamers die *in vivo*-achtige kweekcondities nabootsen tot de ontwikkeling van GMP-compatibele alternatieven voor de componenten die momenteel worden gebruikt in de *in vitro* hCEC-cultuur. Verder is het nodig om te komen tot een betere identificatie van hCEC die wordt gebruikt voor klinische toepassing door middel van fluorescence-activated cell-sorting (FACS) analyse,[24] analyse van het cytokine-niveau[86] en van het transcriptoom, om interindividuele verschillen tussen donoren beter in kaart te kunnen brengen.[87] Parallel hieraan is meer inspanning nodig bij het zoeken naar de "ideale" drager voor hCECs, die een stevige steun

moet garanderen voor gekweekte hCEC, met biomechanische en biocompatibele eigenschappen die vergelijkbaar zijn met de membraan van Descemet. De optimalisatie van deze onderzoekslijnen zal het mogelijk maken om de cornea endotheel cellentransplantatie naar de kliniek te brengen. Dit kan bijdragen aan het overwinnen van het wereldwijde tekort aan donorweefsel en aan het implementeren van een patiëntspecifieke behandeling voor miljoenen mensen die getroffen zijn door cornea endotheel aandoeningen.

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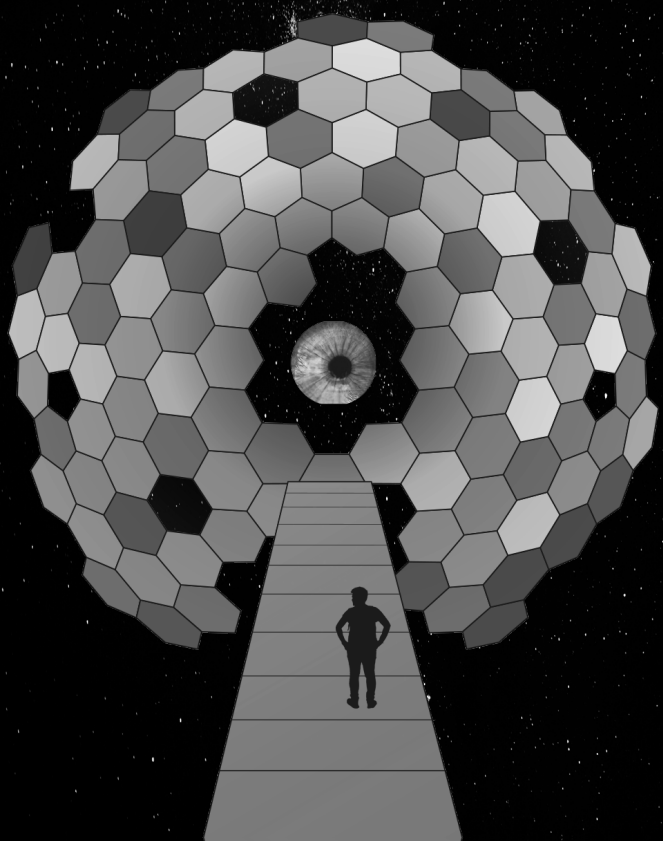
APPENDICES

List of publications

Curriculum Vitae

Acknowledgements



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1. **Spinozzi D,** Miron A, Bruinsma M, Dapena I, Kocaba V, Jager MJ, Melles GRJ, Ní Dhubhghaill S, Oellerich S. New Developments In Corneal Endothelial Cell Replacement. *Review. Submitted for publication.*

2. **Spinozzi D,** Miron A, Lie J, Oellerich S, Ní Dhubhghaill S, Melles GRJ. The influence of preparation and storage time on endothelial cells in Quarter-Descemet membrane endothelial keratoplasty (Quarter-DMEK) grafts *in vitro. Cell Tissue Bank. 2020 Aug 14. doi: 10.1007/s10561-020-09854-z.*

3. Miron A, **Spinozzi D,** Lie J, Melles GR, Oellerich S, Ní Dhubhghaill S. Improving Endothelial Explant Tissue Culture by Novel Thermoresponsive Cell Culture System. *Curr Eye Res. 2020 Jul 29:1-4. doi: 10.1080/02713683.2020.1798468.*

4. **Spinozzi D,** Miron A, Lie JT, Rafat M, Lagali N, Melles GRJ, Dhubhghaill SN, Dapena I, Oellerich S. *In vitro* Evaluation and Transplantation of Human Corneal Endothelial Cells Cultured on Biocompatible Carriers. *Cell Transplant. 2020 Jan-Dec;29:963689720923577. doi: 10.1177/0963689720923577.*

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CURRICULUM VITAE

Daniele Spinozzi was born on May 14th, 1990 in Giulianova (Italy). After graduating from secondary school in 2008 (Liceo Scientifico Marie Curie, Giulianova, Italy), he worked for a Bachelor's degree in Biological Sciences at the Università degli Studi dell'Aquila (Italy). During this training, he did an internship in clinical biochemistry and microbiology at Laboratorio Italia S.r.l. (Giulianova, Italy). He obtained his Bachelor's degree in 2011 with a thesis on the molecular mechanisms underlying trichomes development in *Arabidopsis Thaliana*.

While attending a Master's degree in Molecular Biology at the Università degli Studi di Parma (Italy), he won an Erasmus scholarship to spend a period abroad at the Université de Lille 1 (France). Here, under the supervision of Prof. Robert-Alain Toillon, he worked at the INSERM U908 lab on growth factor signaling in breast cancer cells, in particular on the characterization of the expression of the tyrosine-kinase receptor TrkB in breast cancer cells. This project served as a thesis for his Master's degree in Molecular Biology, which he completed in 2014.

In January 2015, for a period of 6 months, he worked at the Nederlands Kanker Instituut (NKI) in Amsterdam (The Netherlands), where he performed research in the division of Molecular Genetics under the supervision of Dr. André Bergman. The aim of his research during this spell was to elucidate the role and the expression of the androgen receptor in prostate cancer associated fibroblasts.

From January 2016 to December 2019, he worked as a PhD candidate at Netherlands Institute of Innovative Ocular Surgery (NIIOS) in Rotterdam (The Netherlands). Under the supervision of Dr. Gerrit Melles, Dr. Isabel Dapena and Dr. Silke Oellerich, he mainly worked on the establishment of a robust, GMP-compliant cell culture protocol for human corneal endothelial cells, as part of the "ARREST BLINDNESS" project funded by Horizon2020 European Union grant. His main tasks were *in vitro* cell culture with human and animal material, tissue engineering, viability and functionality assays and immunofluorescence. Moreover, in August 2016, he started at the Graduate School at Leiden University Medical Center (LUMC) in Leiden (The Netherlands) as a PhD student in Ophthalmology, under the supervision of Prof. Martine Jager.

Since January 2020, he is working as a PostDoc researcher at the "DRAPH" project on the decellularization and repopulation of aortic and pulmonary heart valves within the Department of Thoracic Surgery at the LUMC, under the supervision of Prof. Mark Hazekamp and Dr. Marten Engelse.

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