Elucidation of the migratory behaviour of the corneal endothelium

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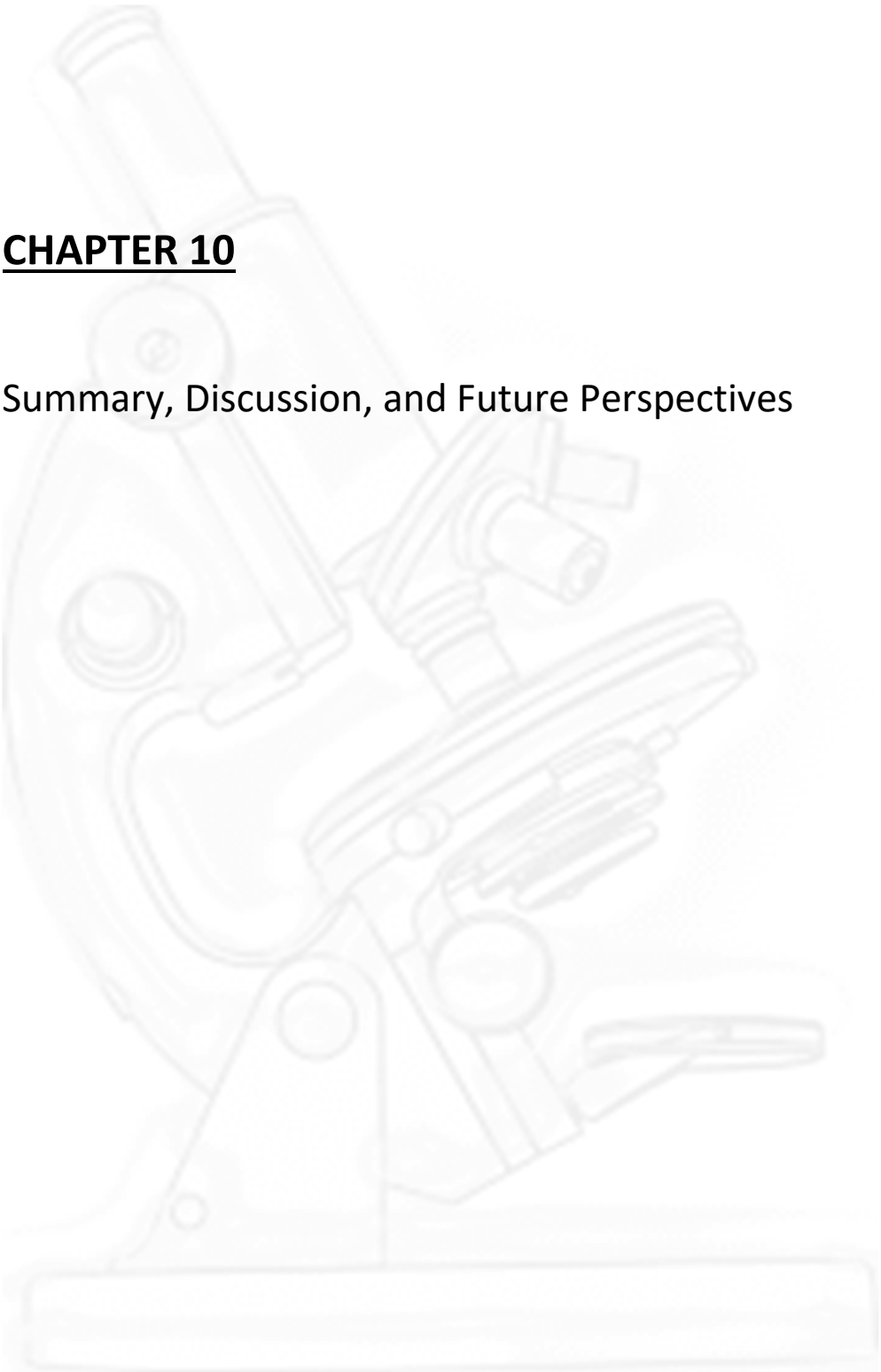
CHAPTER 10

Summary, Discussion, and Future Perspectives



SUMMARY AND DISCUSSION

An intact corneal endothelium is essential for corneal transparency as it regulates corneal nutrition and hydration by balancing a semipermeable barrier activity with active ion transport mechanisms. The human endothelium is thought to be an amitotic cell layer with a continuous and age-dependent loss of endothelial cells of about 0.5 to 0.9% annually.[1] However, the endothelial cell loss can be accelerated due to corneal diseases, damage by inflammatory processes or by mechanical trauma following intraocular surgery or penetrating injury. Although the corneal endothelial cells (CEC) in low density conditions will display increased cellular migration by decreasing contact inhibition, once the endothelial cell density (ECD) drops below a minimum required to maintain the pumping function of the endothelium (typically around 400 – 500 cells/mm^2), it will result in corneal decompensation. In such cases, the damaged or diseased portion of the cornea is surgically replaced either by a full thickness corneal graft (Penetrating Keratoplasty – PK) or a lamellar endothelial cell layer graft (lamellar endothelial keratoplasty – EK). Descemet Membrane Endothelial Keratoplasty (DMEK) is the most selective EK technique and nowadays the preferred treatment option for endothelial diseases.

Similar to solid organ grafting, transplanted corneal tissue possesses a limited lifespan that is often related to the density of cells transplanted. Grafts may display an acute (related to surgical technique or graft preparation) or chronic endothelial cell loss (subclinical immunological reaction) that could lead to graft failure. Moreover, due to the global deficit of donor corneas it is estimated that only 1 in 70 visually impaired patients that require a corneal graft actually receive one.[2,3] In an effort to overcome tissue shortage, Hemi-[4–6] and Quarter-DMEK[7–10] were developed to use the available donor tissue more efficiently. These techniques, like other new treatment options to alleviate tissue shortage, are most appropriate for patients with still healthy peripheral endothelial cells. Therefore, regeneration of the corneal endothelium by tissue engineering techniques, administration of pharmacological modulators or synthetic alternatives is being researched to overcome these problems.[11]

This thesis outlines the rapid progression of the corneal regeneration field, including an in-depth analysis of wound healing pathways and biological modulators. In addition, *in vitro* studies were conducted to evaluate the migration capacity of corneal endothelium before and after EK. These findings result in a better understanding of endothelial cell migration and provided further knowledge for the ongoing research on endothelial graft substitutes.

Early postoperative decrease in ECD after DMEK and DMEK graft viability prior to transplantation

DMEK has become the gold standard to treat endothelial dysfunction owing to the rapid visual rehabilitation, near-normal anatomical restoration of the cornea and a lower risk of allograft rejection.[12] Initially, the DMEK technique was met with some reluctance as there were concerns regarding the technical aspects of graft preparation and surgery.[13] Preparation of thin (10–15 µm) grafts can be challenging and, together with intraoperative graft handling, could potentially lead to either complete tissue loss especially during preparation or to high postoperative endothelial cells loss and low graft survival rate.[14] Since ECD is fundamentally tied to the longevity of endothelial keratoplasty, ECD decrease is considered one of the main outcome measures in the investigation of the efficacy and safety of DMEK, as well as for predicting long-term graft survival.[14–16] Postoperative ECD decrease for all endothelial keratoplasty techniques is usually reported for the six-month follow-up and shows a drop of about 30–40%, comparative to preoperative values, followed by an annual decrease of 7–9% thereafter.[17,18] However, it is unclear at what time point the decrease in ECD reported at six months actually occurred, and whether it reflects a gradual decrease or a sudden drop. The results of a small

case series at our institute showed a larger than 30% decrease in ECD within the first month after DMEK.[19] This finding was substantiated in a follow-up study (**Chapter 1**) on a series of 24 DMEK eyes operated for Fuchs endothelial cell dystrophy (FECD). In this study we were able to obtain specular microscopy images already 1 day and 1 week postoperatively due to the fast corneal clearance after DMEK and we could show that the 30% ECD drop occurs within the first postoperative week; about 2/3 of the total decrease could already be observed after the first postoperative day.[20] Such a rapid decrease cannot be explained by endothelial cell migration and/or redistribution that usually requires more time.[21] Similarly, it is unlikely that such an early drop was caused by an immune response, especially because an allograft rejection is generally considered to be a delayed reaction.[22] Other possibilities for causing the sudden early onset drop in ECD after DMEK may be intraoperative handling or an overestimation of preoperative viable cells on the graft. Since for most surgeries in this study no intraoperative complications were reported, the larger portion of the ECD decrease within the early postoperative phase after DMEK may primarily be explained by the overestimation of the eye bank viable ECD. This led us to examine endothelial cell viability after graft preparation in more detail.

Evaluation of the cell viability and quality of endothelial grafts prepared in the eye bank has become the subject of numerous studies. Endothelial cell loss was evaluated following various graft preparation methods or surgical manipulations.[23–29] Current eye banking practices determine ECD based on the structural integrity of the cells (assessed by trypan blue staining) though this does not exactly reflect the viable cell pool of corneal endothelium allocated for transplantation. Our follow-up study (**Chapter 2**) on graft viability using surgery grade DMEK grafts that could not be allocated (due to the Covid-19-related cancellation of elective surgeries), demonstrated the need to perform a more accurate post-processing corneal endothelial cell analysis.[30] Ideally, grafts should not only be evaluated based on live-dead analysis, but it should be differentiated between various forms of cell death (apoptosis, necrosis, autophagy) since otherwise e.g. apoptotic cells are considered as 'live' cells. For a better differentiation, multiple biochemical and functional assays should be applied. In this regard, Calcein acetoxymethyl ester (Calcein-AM) has been used for studies of enzymatic activity, cell membrane integrity, and long-term cell tracking due to its low cellular toxicity.[31,32] In our study, the cell viability of five grafts scheduled for transplantation was assessed by Calcein-AM on the originally planned surgery day and revealed that the percentage of central surface area covered by viable cells ranged from 57% to 97%. Because of this scattered viability range, we continued with the viability analysis of 11 paired donor corneas evaluated either directly post-preparation or after 3–7 days of storage. Our results showed that cell viability of most DMEK grafts seems not to be affected by preparation and storage, while for some grafts endothelial cell damage undetected by trypan blue could be observed within hours after graft preparation. Because trypan blue can only identify dead cells, it fails to detect apoptotic or necrotic cells.[33] Therefore, when ECD was evaluated after graft preparation by trypan blue staining (eye bank procedure), we observed an average ECD difference of 10 (±21)% compared to ECD determined on the same grafts by Calcein-AM. This large variability of endothelial cell loss observed by Calcein-AM after graft preparation supported our clinical observation that the high endothelial cell loss detected in the early postoperative phase after DMEK can be primarily explained by an overestimation of the graft's viable endothelial cell population.

As an alternative to Calcein-AM, an annexin V-FITC assay has also been reported to detect early apoptosis by targeting negatively-charged phosphatidylserine translocated from the inner membrane leaflet of viable cells to the outer membrane surface during apoptosis.[34] Therefore, combining assays routinely used to characterize cell apoptosis with membrane impermeable dyes such as trypan blue would allow for the detection and quantification, in the same sample, of the apoptotic/necrotic and viable cell populations. However, these assays are not yet approved for the use on transplantable tissue and therefore, there is a still a strong need to develop and validate cell viability and cytotoxicity detection methods that analyze the functional status of the corneal endothelial cells after graft preparation and provide an accurate cell count. Meanwhile, an additional DMEK quality check by light microscopy performed within hour after graft preparation or just before surgery

could help to detect grafts with doubtful endothelial quality and thus, reduce postoperative DMEK complications and low postoperative ECD outcomes.

Perceiving the morphological changes and regenerative capacity of the corneal endothelium *in vivo* and *in vitro*

Intracellular signaling in wound healing

Next to improving the quality of the available corneal donor tissue, current research is also focusing on non-surgical treatments for restoration of corneal endothelium by first understanding the concepts and limitations of clinical procedures. In this regard, the extensive review (**Chapter 3**) on signaling pathways involved in CEC proliferation and migration could lead to new ideas on how to treat corneal endothelial dysfunction in the future.[35]

Developing novel strategies to re-activate CEC regenerative capacity is challenging as CEC are blocked in the G0/G1 phase of the cell cycle *in vivo* and this is further hindered by endothelial-to-mesenchymal transition (EMT). Literature and gene and genome data base analysis revealed a complex interplay of pathways regulating the cell cycle and migration including among others the β-catenin and transforming growth factor (TGF-β) pathways, the PI3K/Akt pathway, and the Rho-ROCK pathway.[36-43] Especially the Rho/ROCK pathway regulates a wide spectrum of fundamental cellular events and is involved in a variety of pathological conditions; its inhibition may trigger various signaling cascades and produce multiple biological effects such as enhanced proliferation, increased motility, or cytoskeleton rearrangements.

In the process of wound repair, corneal endothelial cells may undergo EMT and transform to fibrogenic myofibroblasts. Myofibroblast generation through EMT is largely modulated by the transforming growth factor β (TGF-β)[44,45] that activates not only Smad signals but also other cytokines/growth factors such as mitogen-activated protein kinase (MAPK), P38MAPK.[46–48] Because migration is a major component of wound healing in the corneal endothelium, strategies to inhibit of the unfavorable EMT of the corneal endothelium should not be accompanied with an impairment of cell migration.

The wound healing process of corneal endothelium considers that cells close the wounded gap mainly by migration and increased cell spreading,[49] while cell division remains very low[34] with cells dividing mostly amitotic with formation of binuclear cells.[50] Successful clinical options for replacing the diseased endothelium include approaches that accelerate endothelial healing and suppress EMT through topical administration of ROCK inhibitor eye drops. There is clear evidence that topical Rock inhibitor administered after removal of non-confluent guttae (Descemet stripping only (DSO))[51,52] or after transplanting a devitalized DM[53] for treating central FECD, sustained cornea clearance, improved endothelial cell density, while overall, cells displayed a better architecture. ROCK inhibitors played also a major role in the clinical trial for injecting cultured human CEC into the anterior chamber of the eye.[54,55]

Corneal endothelial cells migrate by transiently acquiring a fibroblast morphology reorganizing the actin into stress fibers, events that are consistent with EMT. Furthermore, EMT may lead to fibrotic complications of healing such as the formation of a retrocorneal fibrous membrane.[56] Inducers of EMT and fibrotic changes in the endothelial layer include interleukine-1 beta (IL-1β) that may be released in response to many 'pathogen associated molecular patterns' (PAMPs) and TGF-β. Although TGF-β can stimulate healing, it also promotes fibrogenic changes including deposition of aberrant extracellular matrix (ECM).[57] To counteract the fibrogenic response, inhibition of TGF-β signaling, viral-mediated overexpression of SMAD7[58] – a natural TGF-β signaling inhibitor, proved to suppress the inhibitory action of TGF-β on cell proliferation, which was mediated by inhibition of SMAD2 phosphorylation and downregulation of p27Kip1.[59] SMAD7 therapy is being currently considered useful for prevention and treatment of fibrogenic disorders in the corneal endothelium.[57]

Clinical scenarios that require corneal endothelial cell migration

Clinical studies on endothelial healing are usually restricted to observations of cases with ocular chemical burn,[60,61] or after replacement of the abnormal corneal endothelium with healthy donor tissue.[62,63] These cases reported corneal recovery either through proliferation of endothelial progenitors from specific regions of the eye (progenitor-enriched niche adjacent to the peripheral endothelium and named inner transition zone (TZ))[64] or combined migration of both donor and remaining recipient endothelial cells. However, the wound healing process of corneal endothelium gives rise to many unanswered questions. For instance, endothelial cell migration insights after Quarter-DMEK surgery (that is, a modified DMEK-technique in which a full-sized DMEK graft is divided in quarters to treat 4 eyes),[8–10] did not succeed in confirming the presence of endothelium progenitors residing in the area close to the limbus. All operated eyes cleared centrally, while the peripheral bare stroma showed persistent edema.[8] The lack of cell migration from that specific region was attributed to the arrangement of collagen fibrillary bands in the graft periphery acting as a barrier for cell migration[6] but could also be caused by the removal of (progenitor) cells during Quarter-DMEK graft preparation. Typical, a DMEK graft diameter of 8–8.5 mm, that is prepared using the no-touch peeling technique, has low chances to show endothelial damage because trephination is performed outside the touched area during preparation.[66,67] However, Quarter-DMEK graft preparation requires manual removal of trabecular meshwork[9] and this technical step may be likely to deteriorate the quality of the peripheral endothelium.

The clinical results of Quarter-DMEK eyes showed a different corneal clearance pattern with clearing primarily occurring adjacent to the radial cut graft edges but not along the 'limbal' round edge of the Quarter-DMEK grafts and in the adjacent bare stromal areas.[8,10] This observation was mainly attributed to an asymmetric endothelial cell migration over different anatomical corneal areas. To better understand the heterogenous cell migration behavior, with migration almost entirely absent in the far periphery of the endothelium, we performed *in vitro* studies to determine how Quarter-DMEK grafts may be positioned best onto the posterior recipient stroma in order to create a more homogeneous corneal clearance pattern (**Chapter 4**).The main experimental challenge was to keep a tissue, inherently inclined to curl, to stay flat in a fixed position on a surface in fluid. While Quarter-DMEK grafts were sandwiched between two glass coverslips spatially separated by a suture wire, the assembly was transferred to a culture plate and cell migration documented over 6 days.[68] Although the experimental set up was rather restrictive for nutrient diffusion, endothelial cells migrated from the radial cut edges but failed from the limbal round edge of the Quarter-DMEK grafts. This finding was mainly attributed to Descemet membrane architecture that organizes the cells in small radial rows induced by the furrow-like distribution of the underlying collagen fibers.[65] Also, it was suggested that endothelial cells undergo, throughout life, a continuous slow centripetal migration from deeper niches toward the center and lose their progenitor phenotype in response to contact with aqueous humor, the presence of TGF-β, and by cell contact inhibition as soon as they form a monolayer.[65] Hence, endothelial cells in the periphery will unlikely migrate outside from the graft area but might still possess residual proliferative capacity.[69,70]

Three-dimensional *in vitro* cell culture model: concept and its applications

Concept description

Given the success to reproduce a clinical observation using an *in vitro* system and research grade donor tissue, we decided to improve the culture technique in order to gather more insight regarding the movement of corneal endothelial cells. But to achieve more reproducible results and reduce the technical burden of the experiments, further optimization of the explant culture system was required. Therefore, we developed a 3D culture technique for explant tissue by using a temperature-reversible hydrogel system which was biocompatible, non-toxic, 100% synthetic, pathogen-free and highly transparent for cell observation (**Chapter 5**). Also, the temperature-dependent dynamic viscosity is an important characteristic that allows the gel to swell, become

soft and flexible upon warming and liquefy upon cooling. This property is very useful to develop methods to harvest cultured cells for specifically planned procedures[71,72] or develop techniques to preserve viable cells within the gel,[73] with no need for an enzymatic treatment.[74] In this study, we expanded the scope of the gel, from an effective culture matrix that provides mechanical support while directing cell adhesion to a structure that adds weight when placed over the top of the biological sample without deteriorating its structure and functionality. In our first *in vitro* cell migration study with Quarter-DMEK grafts sandwiched between two glass slides cell migration could be studied for about 7 days before cells died due to the insufficient supply of nutrients (**Chapter 4**).[68] The new adapted 3D explant culture protocol improved cell viability and collective cell migration continued far longer (>3 weeks).[75] Also, the gel's thermo-reversibility allowed the removal of liquefied gel and enabled the detection of biomolecular markers in the tissue and migrated cell layer which was not possible with the previous experimental set-up.

Study the capacity to induce CEC mitosis in the peripheral corneal endothelium, via the controlled disruption of contact inhibition

Given the advantages of the new 3D culture method to enhance the viability and migration capacity of cells from explant tissue, we continued with testing the effect of different types of peripheral Quarter-DMEK graft modifications on endothelial cell migration (**Chapter 6**). The objective of the study was to further optimize the Quarter-DMEK preparation in order to accelerate corneal clearance in patients along the round edge of the graft. Quarter-DMEK grafts with intact and viable endothelial cells were embedded in a cooled biocompatible, temperature-reversible polymer matrix and cultured over two weeks in a humidified atmosphere.[76] The peripheral edge of Quarter-DMEK grafts was physically modified by either introducing radial cuts into the far peripheral area or by removing parts of the far periphery with a trephine. Immunohistochemistry analysis performed after the two-week culture on grafts retrieved from the polymer matrix, demonstrated the presence of tightly packed and viable cells that showed high migratory ability at the leading edge of the monolayers formed from the radial cut graft edges.

Next to better understanding the molecular pathways involved in endothelial migration (**Chapter 3**), current research also focuses on understanding the structure-function relationships in the adhesive structures of an endothelial monolayer that enable the cell to exert traction on its environment.[77,78] Cells spreading is a process largely determined by two interdependent and interactive systems: the integrin-based apparatus for substrate adhesion and the actin cytoskeleton characterized by distinct arrangements of actin filaments.[79–81] Integrins and actin are coupled through a physical linkage, which provides traction for migration. In collective cell migration cells carry out specialized functions according to their position within the group. Front-rear polarization is an example in which one subset of leader cells at the front guides a larger group of follower cells at the rear.[82] Leader cells typically exhibit a mesenchymal migration phenotype and function by degrading and remodeling the ECM to create channels for the whole cell group to advance cohesively.[83,84] By contrast, followers retain endothelial features such as apical-basolateral polarity and tight junctions and express relatively low levels of guidance receptors. Importantly, cells are not dragged or pushed by neighbors, but actively sense and respond to stresses imposed on them.

Endothelial cell migration from the limbal graft edge, however, was not triggered by increasing cell exposure to free space through surgical modifications of the far periphery. Lack of migration from this area was also not due to an absence of viable cells, since immunolocalization showed cells with expression of structural (zonula occludens-1 (ZO-1) and vimentin) and functional markers (sodium/potassium pump (Na^+/K^+ –ATPase)). At first instance, the furrowed collagen microstructure of the peripheral cornea[65] might have acted as a barrier, thereby preventing migration. At the same time, other stimulus-specific gene expression responses might be required in order to prompt these cells to move. It is possible that important factors responsible for regulating cell migration such as cell-matrix adhesion molecules (e.g., integrins, selectins, cadherins), the Rho family of

small GTPases, and proteases (matrix-metallo proteases (MMPs)), are less expressed in the peripheral cells. When functional integrins recognize ECM ligands (fibronectin, laminin) to form focal adhesion,[85] signaling proteins are recruited to focal adhesion to regulate their assembly and disassembly.[86] Rho family of small GTPases[87] have been reported as key regulators of focal adhesion dynamics by dictating contact association, maturation, and turn over. The disassembly process through which cell adhesions are resealed could be mediated by ECM degradation by MMPs[88,89] or cellular contractile machinery ,i.e., Rho and myosin II, that cause cell rear detachment.[90,91] Taken together, cell migration framework outlines a complex map of processes, with multiple cross-talks between members of different families that influence the cell movement through mutually antagonistic pathways.[92]

Study the regenerative potential of the peripheral corneal endothelium

The failure of far peripheral EC to migrate, in spite of limbal area modifications, still limits the clinical application of Quarter-DMEK. Understanding the nature of these peripheral endothelial cells, how they differ from the central cells, and how to encourage them to migrate would greatly improve the pool of donor tissue available for patients with an immediate need of a transplant.

After having explored the controlled mechanical perturbation of the peripheral endothelium as a possible promoter of collective cell migration, we performed an *in vitro* study to evaluate the potential of ROCK-inhibitor to stimulate peripheral endothelial cell migration. We first adapted the explant tissue shape from a pizza-pie (Quarter-DMEK) to an open-ring (6.5 mm punched out endothelium with TM still attached) to create a better system model for mimicking *in vitro* the *in vivo* effect of ROCK-inhibitor on cell migration outcomes after placing a pie-shaped Quarter-DMEK graft in a circular descemetorhexis area or after DSO (**Chapter 7**). The curved outer graft rims were mounted flat on a substrate, a central prerequisite for observing cell motility, and were cultured in a 3D thermo-reversible hydrogel matrix for over a month. This enabled the assessment whether continuous ROCK-inhibition creates long-term alteration in the migration characteristics of corneal endothelial cells. Our results, described in **Chapter 7,** showed that all cultured rims remained viable and displayed either single regions or collective areas of cell migration, regardless of the presence or absence of a ROCK-inhibitor. Rock-inhibitor, on the other hand, seemed to enhance the morphological stability of the migrated cells. Interestingly, late-onset cell migration from an area close to the limbus was observed. These late-onset cells grew fast into a contact inhibited monolayer displaying the typical hexagonal cell morphology, first adopted a fibroblast-like morphology before acquiring a cell phenotype with a regular morphology and appeared less differentiated compared to other areas of migration. This late-onset cell population not only showed high proliferative capacity but also emerged from outer rim grafts cultured without dysregulating the Rho-ROCK pathway. While it did not alter the cell outgrowth from the outer graft rims, the presence of ROCK-inhibitor did appear beneficial for maintaining the cell shape and cell-cell adhesion contacts during collective migration. The ability of ROCK-inhibitor to promote corneal endothelial wound healing by enhancing endothelial remodeling, adhesion and cell migration has been reported previously.[92]

The broad range of cell migration phenotypes, from non-invasive motility to single-cell mesenchymal style to collective motility, differed in this study compared to previous migration studies of Quarter-DMEK grafts (**Chapter 6**),[76]. The main distinctions in the experimental design were presence of TM that remained adhered to endothelium and cell motility study-period that was considerably longer than two weeks. Thus, it is possible that a particular cell type localized at the insert region of the TM required long-term culture before displaying the characteristics of undifferentiated cells. When cell viability was evaluated on outer graft rims with the late-onset cell population, the intensity of Calcein-AM signal varied over the sample wherein the lowest signal intensity corresponded to the cell population that had emerged from the far periphery of the endothelium. We suspect that this late-onset but fast growing cell population has a low intracellular esterase activity that does not signal damaged membranes but rather low-level expression of esterase-specific genes that serves as a

reliable indicator of undifferentiated cells.[93] Similar to our explant culture, Zhang et al.[94] demonstrated that cells proliferated from peripheral corneal areas with similar morphological characteristics during cell growth, timing, and end cell morphology. Furthermore, by using quantitative polymerase chain reaction (q-PCR), the cultured cells in their study were initially found to express increased levels of stem cell genes and minimal levels of pluripotency but these gene expression levels were reversed later during culture. The conclusion was that cells residing in the Schwalbe's ring region, a transition area between the peripheral corneal endothelium and the anterior non-filtering portion of the TM (collectively called the 'transition zone' [TZ]) displayed characteristics of adult stem cells.

Generally, these cells seem to form a distinct cell population in the transition area displaying distinct ultrastructural features and with a whorled-like pattern oriented circumferentially at the corneal periphery and deep to the corneal endothelium lining of the anterior chamber.[95] Although they were proposed to have neuroregulatory function in the anterior segment [96] they were also found responsible for the formation of an aberrant endothelial membrane covering the anterior uveal meshwork in some patients treated for glaucoma with argon laser trabeculoplasty (ALT).[97,98] In addition, increased cell density in the peripheral areas of the cornea compared to the central area (average range 17%–23%)[99] also suggests that stem-like cells may be present in the peripheral transition region to provide differentiated CEC. Also, it has been documented in the literature that under some circumstances mitosis occurs in the endothelium of the adult human cornea[100,101] and percentage of replication-competent cells is higher in the peripheral CEC than those in the central cornea, which was independent of donor age.[102] These findings suggest that peripheral CEC possess regenerative capacity and may be able to supply new cells for the corneal endothelium. Although molecular marker studies for the stem cell niche at the transition zone provide supportive data,[103,104] there has not been a stem cell signature established so far.[105] Also, attempts to isolate and propagate undifferentiated progenitor cells using a sphere culture protocol have proved to be more effective in isolating young precursor cells[106] from the peripheral corneal endothelium than the central region.[107–110] Therefore, it still remains to be determined if the Schwalbe's cells, TZ cells, and precursors are the same cell type, the extent to which they retain regenerative potential, and how cellular proliferation could be unlocked *in vivo* to repopulate corneal endothelium in age and disease.

Improving surgical technique by integrating *in vitro* cell culture observations

While trying to understand and promote EC migration from the peripheral cornea, the low postoperative ECD after Quarter-DMEK helped us to focus on continuous technique improvement. In an effort to address the significant ECD decrease after Quarter-DMEK,[8,10] which was thought to be caused by the shape mismatch between a round descemetorhexis and a triangular graft, a new surgical option was described, where small diameter DMEK grafts were prepared to match a small descemetorhexis and validated through a series of *in vitro* experimental conditions (**Chapter 8**). The main findings of this study were: (1) three circular mini-DMEK grafts with a diameter of 4 mm can be successfully prepared from one donor cornea, (2) the surgical procedure could be validated *in vitro*, and (3) small-diameter grafts embedded into a thermo-responsive hydrogel matrix showed uniform cell migration around the entire circular graft edge with cells displaying typical hexagonal close-packed morphology.[111] Similar to Quarter-DMEK, transplantation of a small-diameter graft offers the theoretical benefit of reduced donor antigen load and may allow using donor corneas with multiple incisional scars following cataract extraction. Initially, grafts as small as the 4 mm diameter (mini-DMEK) were reported to treat acute corneal hydrops in keratoconus (i.e., rupture and detachment of the stiff DM due to progressing ectasia of the corneal stroma).[112,113] Not only the shape and size of the DMEK grafts used to close the tear in the DM were not standardized (5 mm round DMEK graft or razor blade cut graft with a width of 3 mm and a length adjusted to the length of the tear in the recipients' DM) but also the orientation of the graft was not important for the surgery, presumably because the healthy host endothelium would easily repopulate the DM

even if the graft was accidentally inverted.[113] In a more recent study, Handel et al.[114] utilized mini-DMEK grafts to treat chronic focal corneal endothelial decompensation caused by tears in Descemet membrane after intraocular surgeries or corneal edema in the area of Haab striae in buphthalmus. Therefore, corneas were healthy and no disease except for the focal DM defect was present. The mini-DMEK grafts were trimmed from remaining DM to a width and a length equal to the length of the tear in the recipient's DM, while the central DM was used for patients with FECD. Although cornea deswelling was observed in all cases, the role of endothelial cells in small DM defects remained unclear.

It should also be noted that the small-diameter DMEK grafts have the potential to increase the use of one donor cornea to benefit three recipients only for treating mild FECD with guttae confined to the 4 mm central area. To avoid the 'no-touch' handling-related challenges of such small DMEK grafts, two alternative methods have been clinically tested so far, namely DSO and transplantation of acellular DM (i.e., Descemet membrane transplantation, DMT).[115–120] DSO represents a donor-independent strategy for central FECD, an approach that has already extensively been discussed in **Chapter 4**, while DMT represents a strategy for using non-clinical grade DMEK tissue. Although both techniques have the potential to treat FECD without the need for allogenic cell transplantation and fear of graft rejection, DMT provides an appropriate substrate that supports host endothelial cell migration with reduced risk for cells to enter endothelial-to-mesenchymal transition.[53] In addition, it seals the stroma to avoid keratocyte activation close to the wound space by the aqueous TGF-β,[121] that may lead to fibrosis and increased risk for retrocorneal membrane formation.[122,123] However, both DSO and DMT report a long recovery time with complete anatomical cornea restoration and visual rehabilitation not earlier than 3 months postoperatively.

Small diameter DMEK grafts showed great surgical feasibility with improved graft characteristics (i.e., ECD, graft viability, uniform cell migration capacity) and by having a matching shape to the circular descemetorhexis, clinical recovery could be similar to conventional DMEK. However, results of clinical tests would provide greater clarity about the efficiency of small diameter-DMEK grafts for treatment of mild FECD.

FUTURE PERSPECTIVES

DMEK is nowadays the gold standard for the treatment of corneal endothelial dysfunction. Since its introduction, DMEK has proved superior to PK and other keratoplasty techniques in terms of faster visual recovery, lower rejection rates, better refractive outcomes, and increased structural integrity.[124–128] Therefore, the number of DMEK procedures performed worldwide has increased, particularly, in patients with Fuchs endothelial corneal dystrophy (FECD).[129,130]

By only replacing the diseased tissue, DMEK embodies conceptual simplicity and surgical sophistication. Nonetheless, the main problem with endothelial keratoplasty is the chronic loss of endothelial cell density (ECD) over time which is similar to PK.[124,131,132] The effect of several donor- and patient-related parameters on endothelial cell loss has been evaluated in several studies in the literature, but with no consistent outcome.[15,133–140] However, the intraocular handling of the 15–20 µm thick membrane and the preoperative manual graft preparation represent technical challenges that may affect the final outcome.

We performed studies to better understand the postoperative ECD decline, as described in this thesis. One aspect regards the overestimation of graft viability in the eye bank,[30] which in turn results in an unrealistic high drop in ECD in the early postoperative phase after DMEK.[20] Grafts seem to develop pronounced endothelial cell damage even after an unremarkable preparation process, However, performing DMEK surgery using tissue with suboptimal endothelium quality could increase the risk of graft detachment and early graft failure.[14] While candidate fluorescent vital dyes can visualize life and apoptotic cells, regulatory and safety concerns as well as economic considerations may prevent eye banks from implementing such a step in their current protocol. One short-term solution could be to check the tissue quality just before releasing the graft for

transplantation. Although implementing this step might lead to an increase in the discard rate of already scarce tissue, it may result in a lower re-transplantation rate. An alternative strategy could be to improve the quality of donated corneas, by boosting the storage media with pharmacological modulators able to promote corneal endothelial regeneration and by maintaining a low level of oxidative stress. Furthermore, storing the cornea in a bioreactor, and not just free floating in a sealed bottle, could recreate the pressure gradient equivalent to intraocular pressure associated with a continuous renewal of storage medium, reduce stromal swelling, and therefore improve EC viability.[141,142] However, further research is needed to evaluate the safety of such storage methods and the therapeutical relevance of pharmaceutical agents.

In an effort to overcome tissue shortage, the use of Quarter-DMEK could potentially quadruple the pool of donor tissue. However, the technique may benefit from some further modification to improve ECD outcomes. *In vitro* studies on the endothelial cell migration included in this thesis showed that the round peripheral edge of the Quarte-DMEK graft will constitute a physical barrier for cell migration[68,76] unless progenitor-like cells, recently discovered in an area close to the limbus,[64] could be unlocked to induce sufficient corneal deturgescence. Also, by adapting the graft preparation protocol to eliminate the round peripheral edge of the Quarter-DMEK, small diameter-DMEK may provide a fast and uniform corneal clearance and become a viable clinical option to treat central endothelial disease.[111]

The limited numbers of high-quality corneal donors, and the surgical complexity of DMEK has promoted significant research interest in developing alternative techniques that either encourage a more efficient use of donor tissue or completely eliminate the need for implanting donor tissue.

To date, no better therapeutic alternatives are available for the treatment of diseased endothelium than corneal transplantation. However, current tissue engineering approaches for corneal replacement represent a promising avenue for clinical applications. To overcome cornea donor shortage, researchers have adopted two basic tissue-engineering approaches: a “cell-based” strategy to allow the cells to create their own extracellular matrix, and “scaffold-based” strategies to provide strong and biocompatible matrices upon which to grow cells.[143–146] Regardless of strategy, *in vitro* expansion or the *de novo* generation of corneal endothelial cells (CEC) from pluripotent stem cells or other cell sources is required.[147,148] The main challenge for the *in vitro* proliferation of terminally differentiated cells is to preserve their phenotype by avoiding endothelial-to-mesenchymal transition (EMT), which can cause CEC to lose their normal cell morphology and induce cell fibrosis. The alternative of differentiating CEC from pluripotent stem cells or other cell sources such as bone marrow-derived endothelial precursors, neural crest cells, corneal stromal stem cells, skin-derived precursors, or mesenchymal stem cells requires suitable culture protocols which have to comply with regulatory directives to guarantee that the final cell source resembles CEC.[148–154] While good manufacturing practice directives may differ depending on the country or region in which they are defined, there is an urgent need for standardization of endpoint parameters that generated CEC should fulfil. Therefore, the list of quality criteria should be reviewed for: (i) morphology assessment by checking cellular hexagonality upon reaching confluence in culture, (ii) genotype and phenotype by examining structural and functional markers, (iii) karyotype conservation by checking the integrity of the DNA to demonstrate the lack of gross chromosomal aberration, and (iv) functionality checked *in vitro* by tools that measure ion permeability across a monolayer of cells, *ex vivo* using corneas in a setting that mimics physiological conditions and allows the measurement of corneal thickness and further correlate to cell functionality, or *in vivo* using animal models of corneal edema.[155]

After facing all challenges with CEC culture in terms of cellular profile, proliferative capacity, and downstream analysis, cells must be delivered alive and with sufficient potential to adhere to the posterior part of the cornea. The “cell-based” strategy proposes the delivery of CEC in a simple and minimal invasive manner via injection into the anterior chamber of the eye.[155] After the procedure, placing the subject in a prone position for 3 hours allows gravity to increase the attachment of CEC to the posterior part of the cornea. The proof-of-concept clinical study by Kinoshita and associates demonstrated that corneal edema could be reversed by injecting about

$1x10^6$ cultured human CEC supplemented with ROCK inhibitor Y-27632 into the anterior chamber after mechanical scrapping of the diseased endothelium; corneal clarity was maintained at least 5 years postoperatively.[54,55] Additionally, the latest technique refinement suggests that injection therapy using highly purified mature cultured human CEC for corneal endothelial failure is safer, provides rapid recovery of corneal thickness, better ECD, and a low cell attrition rate over 3 years postsurgery.[156] However, larger, prospective, randomised controlled trials are required to ensure the long term efficacy and safety.

The main challenge for the "scaffold-based" strategy is to obtain a monolayer of CEC on a biocompatible carrier to produce bioengineered corneal endothelial grafts.[145] The use of a carrier that supports cell replication is an attractive approach because it has the added advantage of delivering a contact-inhibited and functional cell monolayer to the correct place and in a controlled manner. In addition, fewer cells are needed to populate the carrier compared to cell injection, thereby increasing the number of patients that could benefit. Assuming a surface area of 57 mm^2 (8.5 mm circular carrier) and a final ECD of 2300 cells/mm^2 (usual threshold value set up by eye banks), an endothelial graft should contain about 1.3×10^5 CEC. Based on a simple calculation, the expanded CECs used to treat 11 patients by cell injection could hypothetically populate 84 carriers and treat patients by a delivery strategy similar to DMEK or DSEK. However, an ideal cell carrier should mimic key architectural and functional features of the DM and therefore be dense, thick enough to provide sufficient mechanical strength, relatively transparent, semi-permeable to aqueous humour, flexible enough to mould to the curvature of the cornea, biocompatible, promote cell adhesion and phenotype, and maybe biodegradable to enables cells to produce their own DM while simultaneously degrading the surrounding scaffold. Many *in vitro* studies have reported promising research results when using either natural tissues such as decellulared biological membranes (e.g., amniotic membrane, denuded DM or stroma of both human and animal origin, human anterior lens capsule)[157–167] or polymeric materials (natural and synthetic).[161,168–192] Subsequent *in vivo* testing of tissue-engineered corneal endothelial cell-carrier sheets in animal models has, however, not proven any of the constructs suitable to progress into clinical practise.[160,193–195]

The option to eliminate the transplant altogether and allow a person's own endothelial cells to redistribute was introduced by Descemet stripping only (DSO) for the treatment of early FECD. In a primary analysis of DSO, the removal of a 6mm diameter area of the diseased DM led to an incomplete recovery.[116,196] Better clearance rates were reported where a smaller 4-5mm descemetorhexis was employed in selected cases of relatively young patients with central guttae and an adequate peripheral endothelial reserve.[116,197] Despite these limitations, DSO benefits from 0% rejection rate (no risk of immunologic graft rejection) and no need to use long term topical corticosteroids to prevent graft rejection thereby reducing the side effect of intraocular pressure elevation. However, DSO is not yet a replacement for DMEK for two primary reasons: clearing a smaller area may still lead to suboptimal vision, and corneal edema may persist for months hindering visual recovery, rendering its outcome unpredictable.[119] To improve its success, this technique may require the use of pharmacological modulators such as Rho-associated protein kinase (ROCK) inhibitors.[118] Although the biological action of ROCK-inhibitors is completely understood, they have been described to significantly speed up the visual recovery and induce higher central endothelial cell counts in a restored endothelium and with an improved cell architecture.[52] Therefore, DSO may be a suitable first-line surgical treatment option prior to DMEK or small diameter-DMEK, for those willing to try if stripping alone will resolve their vision problems. However, larger trials are still required to assess the effect of DSO in conjunction with pharmacological drugs on longer-term clinical efficacy and drug safety.

A potential hybrid technique between DSO and conventional, circular DMEK employs the use of endothelial graft substitutes comprising of tissue-derived or synthetic matrices.[120,198,199] Transplantation of an acellular DM into a patient has recently been reported as part of a larger clinical trial in Singapore [identification number NCT03275896]. The patient was transplanted with a 4 mm decellularized membrane and showed a four-line improvement in acuity 6 month after transplant, with near to normal restauration of central corneal

thickness and ECD values comparable to DSO.[120] Alternatively, a synthetic graft substitute (EndoArt) has been implanted to reverse corneal enema and promote sight recovery.[198] Attached to the back of the cornea, EndoArt should prevent the transfer of fluids into the cornea and inhibit the fluid accumulation leading to edema. A summary of the first results for two patients as part of a multi-center, prospective feasibility clinical study [identification number NCT03069521] showed that patients had a reduction of corneal edema with transparency recovery after EndoArt implantation. Limitations of implanting this synthetic construct include: (i) regular repositioning by rebubbling until complete adherence to the stromal bed, (ii) unclear timespan over which the cornea will remain transparent and properly hydrated, (iii) long-term effect of restricting diffusion of vitamin and essential nutrients from the aqueous humour to the cornea and (iv) inability of corneal endothelial cells to migrate and populate the artificial layer. Overall, natural-derived or engineered graft substitutes will still have to be evaluated in large clinical trials with long-term follow-up results to further determine their implementation success and also identify the right target populations.

Another strategy to increase corneal endothelial graft availability is to treat the genetic disorder by replacing the need for a corneal transplant. The current strategies able to correct the genetic alteration or avoid their associated effects are gene augmentation therapy (GAT), antisense oligonucleotide-based modulation (AON), and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9)-based modulation.[200–205] It has also been reported that FED pathophysiology manifests through a combination of various genetic and non-heritable factors, such as channel dysfunction (e.g., solute carrier family 4 member 11 – SLC4A11), abnormal extracellular matrix deposition (e.g., collagen type VIII alpha 2 chain – COL8A2), RNA toxicity, oxidative stress (e.g., nuclear factor, erythroid 2 like 2 transcription factor – NRF2), and apoptosis (e.g., zinc finger e-box binding homeobox 1 – ZEB1).[206,207] The most common genetic alteration in FECD is a microsatellite region comprising CTG trinucleotide repeats (TNRs) in the fourth intron of the TCF4 gene to be abnormally expanded and segregated. While the genetic mechanism responsible for the effect of this trinucleotide expansion on the TCF4 gene is unclear, it will contribute to cellular dysfunction by triggering RNA mis-splicing. The genetic modulation of TCF4 expression is done either by transferring a functioning copy of this defective gene aimed to correct the disease, by introducing antisense oligonucleotides such as small interference RNA (siRNA) or micro-RNA (miRNA) that could diminish the toxic effects associated with the defective gene, or by eliminating the CTG expansion in order to revert the mutation causing FECD.[208–214] Further research is also needed to explore the immune tolerance towards the transgene products following repeated administration in the anterior eye chamber, find the most efficient and cost-effective delivery methods, and identity the off-target effects.

Over the past several years, the use of pharmaceutical agents for the treatment of corneal endothelial diseases has been explored.[35] The working principle relies on promoting cell survival, proliferation, and migration with a minimally invasive approach of intracameral or topical drug delivery. ROCK-inhibitors have been the most studied drugs with great potential to trigger CEC repair *in vivo* in humans when administrated topically as an adjuvant to DSO.[51,52] Worldwide clinical series report on ROCK-inhibitors success to reverse corneal edema after surgical removal of diseased CEC, restore corneal anatomy after partially detached DM in BK eyes after cataract surgery, and regenerate the corneal endothelium through a presumed increase in cell proliferation.[118,215–217] Also, promising research has been reported for other pharmaceutical drugs such as epidermal growth factor, platelet-derived growth factor, or fibroblast growth factors.[218–220] However, they should be administered with caution as they show a dual mechanism of action, i.e., regeneration potential with the risk of causing an undesired EMT. Attention has also been directed to reduce oxidative stress by upregulating transcription factors to promote the expression of antioxidative stress proteins thereby decreasing CEC apoptosis.[221–226] Also, profiling new drug candidates require a systematic examination of the functional effect in a variety of *in vitro* and *in vivo* assays. Furthermore, patient assignment in a clinical trial requires extensive knowledge on the diseases to be treated. In order to conclude any beneficial effects of the drug candidates mentioned above, it is mandatory to perform large, randomized control trials to generate higher level evidence.

Conclusive remarks

Despite significant progress towards therapies to promote corneal endothelial regeneration, there is still a long way before such therapies are approved by regulatory bodies and become routine clinical practice. To date, replacing the diseased endothelium by DMEK is still the most efficient treatment option for endothelial dysfunction, but the number of procedures is still restricted by a worldwide shortage of suitable and available human donors, especially in resource-poor parts of the world. Moreover, considering the COVID-19 pandemic, tissue exclusion criteria have become even more stringent, limiting considerably the pool of available donors.[227] It is essential to make the added value of the donation process clear to people, to have an incentive to register for donation because they are more likely to gain from the system than to contribute to it,[228–230] while in the meantime new treatment options are being developed and translated into clinical practice.

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