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Elucidation of the migratory behaviour of the corneal endothelium

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Citation

Miron, A. (2023, March 9). *Elucidation of the migratory behaviour of the corneal endothelium*. Retrieved from <https://hdl.handle.net/1887/3570514>

Version: Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).



CHAPTER 1

Introduction and outline of the thesis

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GENERAL INTRODUCTION

Posterior lamellar keratoplasty techniques have gained increasing acceptance over the past two decades to become the leading form of corneal transplantation. Initially described by Melles et al in 1998, there have been multiple technical modifications, culminating in Descemet membrane endothelial keratoplasty (DMEK).[1,2] These technical refinements have allowed endothelial keratoplasty to gain not only widespread acceptance but to make it the treatment of choice for endothelial diseases.[3,4] The selective transplantation of the endothelium through a small incision allows for faster and more complete visual rehabilitation, with minimized interface haze and antigen load, less astigmatism and fewer suture-related complications.[5,6] Fuchs endothelial corneal dystrophy is the most common indication for corneal transplantation.[7,8] It is characterized by deterioration of endothelial cells, from the center towards the corneal periphery, and the development of characteristic basement membrane excrescences known as guttae. Interestingly, the diseased endothelial cells have demonstrated an increased migration speed compared to normal corneal endothelial cells, which may impact the healing response to surgical procedures under pathological conditions.[9]

The research presented in this thesis focusses on *in vivo* and *in vitro* corneal endothelial cell migration from shape-adapted corneal grafts for the treatment of corneal endothelial disorders. A greater understanding of the cell migration mechanisms from phenotypically distinct regions of the endothelium may assist in selecting the optimal donor tissue and predicting *in vivo* cell behavior. Understanding cell migration *in vivo* has clinical implications for corneal transplantation, for instance when pharmaceutical cell modulation may further improve the patient outcomes. In this introduction, we will discuss the corneal anatomy, the basic physiological function of the corneal endothelium, surgical approaches, and available treatment options for mitigating endothelial cell dysfunction.

THE HUMAN EYE

The eye consists of three basic structural layers, which enclose the optically clear aqueous humour, lens, and vitreous body. The outermost layer is a tough collagenous structure comprised of the cornea and the sclera. The middle layer is highly vascular and is known as the uvea. This layer contains the main blood supply to the eye and consists, from posterior to anterior, of the choroid, the ciliary body, and the iris. The innermost layer is the retina, which rests on the choroid and lines the inside of the posterior segment. The retina is one of the most metabolically active tissues in the body, receives most of its nourishment from the vessels within the choroid, and is responsible for the perception of images (Figure 1). To reach the retina, light must pass through and be refracted by the cornea which is responsible for two thirds of the refractive power of the eye.

The Cornea

The cornea is a transparent avascular tissue about 520 μm thick which acts as a primary barrier against infection and mechanical damage to the internal structure of the eye.[10] The role of the cornea in the refraction of light requires it to be both optically transparent and sufficiently curved to bend light rays with minimal light scattering. Normally, more than 90% of the incident light is transmitted through the cornea.[11] Maintaining corneal transparency is of prime importance for visual function. Since the cornea lacks blood vessels, the anterior surface receives nutrients via diffusion from the tear fluid, the periphery from scleral vessels while the posterior side is supplied by the aqueous humor. The cornea is also supplied by neurotrophins via the nerve fibers that innervate it. Its anatomic structure is relatively simple consisting of three cellular layers, namely the epithelium, the stroma and the endothelium which contain epithelial cells, keratocytes and endothelial cells, respectively. In addition, there are two important acellular interfaces: the Bowman layer between the

epithelium and the stroma, and the Descemet membrane (DM) between the stroma and endothelium (Figure 2).

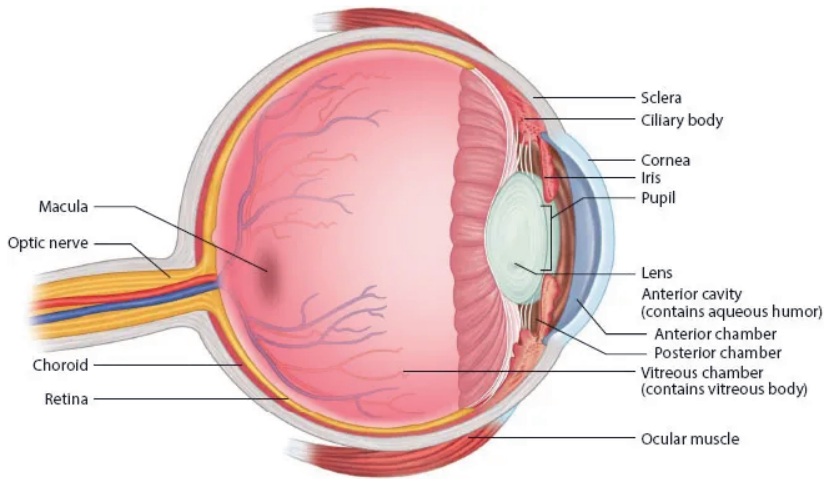


Figure 1 | Anatomy of the eye – sagittal section.

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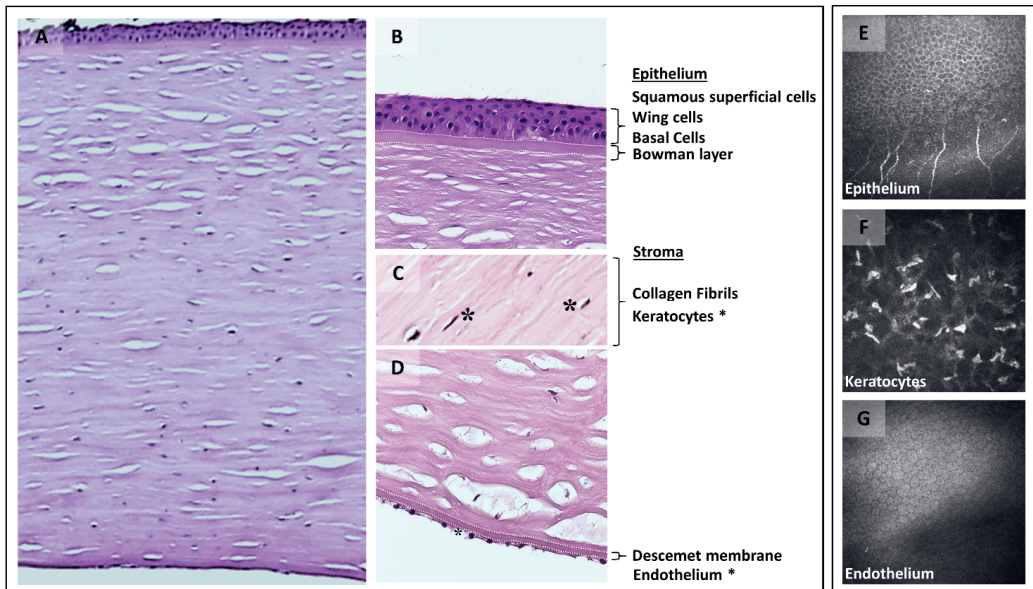


Figure 2 | The human cornea. (A) Photomicrograph of a cornea stained with hematoxylin-eosin (H&E) with emphasis on (B) epithelium – formed by superficial squamous cells, wing cells and a single layer of inner columnar basal cells, Bowman layer – an acellular tough membrane situated between corneal epithelium and stroma, (C) stroma – the layer that gives the cornea strength and gives it its curved shape, (D) Descemet membrane – the innermost surface of the cornea that acts as a basement membrane for the inner endothelium – a monolayer of homogeneous, closely packed, hexagonal cells; original magnification: (A) 100 μm , (B-D) 400 μm . (E-G) confocal microscopy images of epithelial cells, stromal keratocytes and endothelial cells.

The epithelium

The epithelium is a five-to-seven layered cell sheet that serves a number of functions. It helps to keep the corneal surface optically smooth and provides a barrier to external biological agents and chemical damage.[12] It represents about 10% (~ 50 μm) of the total corneal thickness and it is constantly sloughed off and regenerated, which helps the eye to heal itself from mild trauma or abrasions. The epithelium consists of flat superficial differentiated cells, deeper winged cells (daughter cells of the basal layer which are pushed anteriorly), and an underlying monolayer of columnar basal cells. The differentiated squamous cells have surface microvilli and occupy the outer 1 – 3 cell layers of the epithelium (Figure 2, A, B, and E). The function of the microvilli is to increase the cell surface area allowing a close association with the tear film. The underlying basal cells have wing-like extensions, rarely undergo division and migrate superficially to differentiate into squamous cells. The innermost basal cell layer consists of a single layer of columnar cells with important functions including the generation of new wing cells and maintenance of the epithelial organization, and acting as a scaffold on which cells can migrate. The transparency of the normal epithelium is the result of the homogeneity of the refractive index of cells throughout this cellular layer.[13] When an excessive accumulation of fluid (edema) occurs, the epithelium loses its homogeneity and the corneal surface becomes irregular. Any surface irregularity can cause a reduction in vision along with symptoms of glare, photophobia, and halos around lights due to light scatter.

The Bowman layer

The Bowman layer forms the anterior boundary between the epithelium and the stroma and consists of randomly oriented collagen type I fibrils supported within a proteoglycan matrix. This interconnecting network of the anchoring fibrils in the anterior cornea confers considerable strength and resistance to trauma and helps the cornea to maintain its shape (Figure 2, A and B). However, once damaged, it cannot be regenerated.[14]

The stroma

The stroma is a structured lattice of collagens and proteoglycans deposited in sheets known as lamellae and maintained by specialized keratocytes (Figure 2, A, C, and F).[15] The keratocytes are scattered throughout the stroma and are linked to one another via dendritic processes.[16] Keratocytes are typically dormant in the quiescent stroma but can become active and then produce and turnover crystalline proteins to maintain corneal transparency.[17] The unique arrangement of evenly-spaced collagen fibrils provides structural strength, shape, stability, and transparency to the cornea.[18]

The Descemet membrane

The Descemet membrane (DM) is a thin acellular layer that acts as the basement membrane of the endothelial cells. It is continuously produced and deposited by the endothelial cells, resulting in a thickness increase throughout life at a rate of about 1 to 2 μm per decade reaching about 10 μm in older adults (Figure 2, A and D).[19,20] In adults, the DM consists of two ultrastructurally distinct layers: an anterior, highly organized banded layer of collagen lamellae and proteoglycans formed during gestation, and a posterior, more amorphous layer produced by the extracellular matrix deposition of the endothelial cells.[21] The gradual increase in thickness of the posterior layer suggests that either there is no degradation of its constituents or the rate of synthesis of constituents is higher than the degradation rate.[22] The wide-spaced collagen fibers found in the DM do not adhere strongly to the stroma, and so a surgical cleavage plane can be created allowing the DM to be peeled and dissected as a sheet.[23]

The endothelium

The endothelium is a thin monolayer of hexagonal cells covering the posterior surface of DM, lining the inner surface of the cornea, and maintained by nutrients from the aqueous humor (Figure 2, A, D, and G). Despite their simple hexagonal appearance, endothelial cells are quite complex in function. Adjacent cells communicate through intracellular junctions[24–27] that mediate electrical and chemical coupling between neighboring cells, whereas the basal cell margins adjacent to DM are ruffled, with tightly-joined interdigitating foot processes, some of which appear to insert into neighboring cells.[28,29] The whole assembly allows the endothelium to function as a “leaky” barrier, forming resistance to the permeability of solutes and fluid through paracellular transport routes,[27,30] but allowing the passage of nutrients from the aqueous humor to feed the avascular cornea.[31] The corneal endothelium (CE) counteracts the osmotic tendency of the corneal stroma to swell by removing excess stromal fluid via the activity of proton pumps, which are located mainly on the basolateral side of the membrane.[32,33] The dynamic balance between the barrier and active pump of the endothelium is essential for maintaining the relatively dehydrated state of the stroma required for transparency (i.e., stromal deturgescence).[34] Once the endothelial monolayer is compromised, the relative balance between the leak rate and metabolic pump rate is lost.

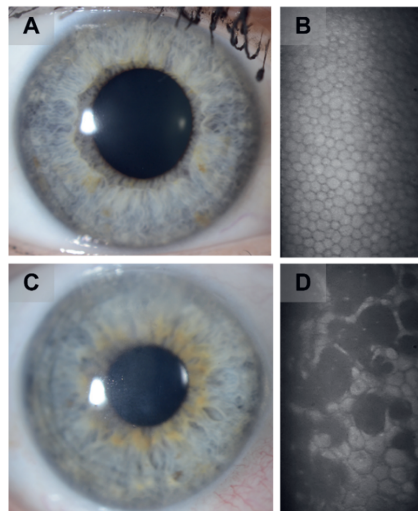


Figure 3 | Slit-lamp photograph showing a transparent cornea (A, slit lamp image overview) optimally hydrated by a healthy endothelium (B, specular microscopy image showing an endothelium characterized by hexagonal cells with highly uniform polygonal morphology). Corneal endothelial decompensation (D, specular microscopy image showing empty spaces in the endothelial mosaic) leading to overhydration of the cornea (C, slit lamp image overview).

Endothelial cell density (ECD) begins at about 5000-6000 cells/mm² at birth, but as the cornea grows the cells spread out and the density declines to about 3500 cells/mm² in the young adult. From the second to the eighth decade, the cell density further decreases to about 2600 cells/mm² with an attrition rate of approximately 0.6% per year.[35–37] Human corneal endothelial cells (hCEC) are not thought to have a significant capacity for *in vivo* regeneration, thus making them unable to replace significant numbers of dead or damaged cells.[38] While the cells do not appear to have the capacity to proliferate, hCEC can respond to minor damage by stretching and centripetal migration into the injured area, to maintain proper structure and function.[26,35] The phenomenon of cell spreading can be associated with the variability in cell size (polymegathism) and cell shape

(pleomorphism) observed in older individuals. Given the importance of its function, damage to the endothelium is potentially more serious than that to the other corneal layers and can result in cell loss and loss of vision.[40] However, the age-associated decline of the CE does not usually affect the critical barrier and pump function.[41] In contrast, acute hCEC loss due to conditions such as endothelial dystrophies, chemical burn, or previous refractive or intraocular surgeries may lead to corneal decompensation.[42–48] When the hCEC density decreases below the critical threshold range of 500 to 1000 cells/mm² the pump function may be compromised, and the cornea can become edematous and cloudy (Figure 3).[49] In such cases, corneal transplantation is currently the only effective option to improve vision and reduce pain. The gold standard treatment is to replace the ineffective endothelium selectively with healthy, functional donor CE through a corneal transplant.

ADVANCES IN ENDOTHELIUM TRANSPLANTATION AND REGENERATION

Cornea transplantation is an operation used to remove all or only the damaged part of the cornea to replace it with healthy cornea tissue (the transplant) of a suitable deceased donor. Fuchs endothelial corneal dystrophy (FECD) is a common form of corneal endothelial dystrophy and the most common indication for cornea transplantation worldwide.[50] FECD dystrophy progresses slowly and is characterized by a progressive loss of central corneal endothelial cells, thickening of DM and deposition of basement membrane excrescences in the form of guttae (Figure 3, C and D).[51]

Before cornea transplantation can be performed, the donor tissue should be harvested, disinfected, assessed, prepared and stored, a highly regulated process most often performed by an eye bank. Since the first successful transplantation in 1905 by Zirm,[52] for many years, the procedure of choice to manage corneal disorders was penetrating keratoplasty (PK), i.e., a full-thickness graft in which all corneal layers are replaced (Figure 4, top). Although improvements in the visual acuity were achieved with PK, frequent complications were reported: (i) denervation, (ii) suture-related complications such as astigmatism, infection and increased risk of immune-mediated graft rejection, (ii) and a significant increase in the prevalence of glaucoma following transplantation.[53] Moreover, since visual acuity outcomes after PK were not predictable, the procedure was usually only performed after the patient’s visual acuity level had dropped to levels below 0.3.[54,55] These drawbacks of PK led to the development of posterior lamellar keratoplasty techniques to replace PK with less invasive surgical interventions for the treatment of endothelium-related corneal diseases.[56–59]

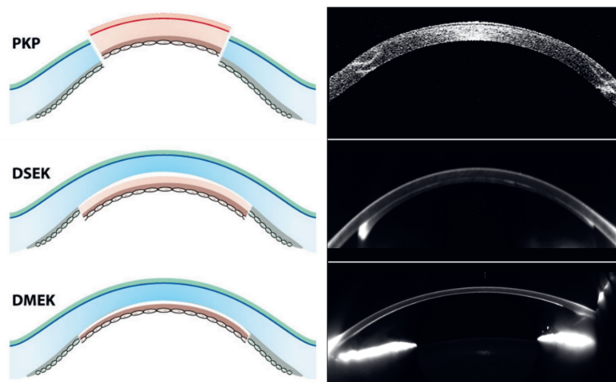


Figure 4| Fundamental developments in endothelial keratoplasty. Cartoon images of the PK, DS(A)EK, and DMEK principles (Left) and the post operative view of the cornea after the three procedures (Scheimpflug image – Pentacam), respectively (Right).[60-62]

One of the keystones of modern endothelial keratoplasty (EK) was laid by Melles et al.[63] in 1998, where he described the posterior lamellar keratoplasty (PLK), an initially mid-stromal approach to replace the posterior cornea. Since the removal of the diseased tissue for PLK was technically still challenging, in 2002, Melles introduced the ‘descemetorhexis’ procedure, during which the diseased host Descemet membrane with its endothelium is selectively removed (‘stripping’).[64] This enabled the introduction of Descemet stripping (automated) endothelial keratoplasty (DS(A)EK), a procedure that became adopted by corneal surgeons all over the world (Figure 4, middle).[65] Compared to PK, DS(A)EK resulted in a faster visual rehabilitation and more predictable refractive outcome, while suture and wound-related problems could be avoided. DS(A)EK allows the retention of a better structural integrity of the eye and allograft rejection rates were reduced.[66,67] However, thickness irregularities of the donor graft or stromal interface haze causing optical aberrations could sometimes result in variable visual acuity outcomes.[67] Following the widespread adoption of DS(A)EK, the Melles group further revised its approach and reported the results of a newer procedure that eliminated the variability of the stroma by producing a stroma-less graft in the form of Descemet Membrane Endothelial Keratoplasty (DMEK).[68–70] In DMEK, the normal corneal anatomy is restored by selective replacement of the diseased corneal layer only (Figure 4, bottom). Although initially challenging, recent developments have facilitated tissue handling in a “no-touch” manner. As a result, the procedure is designed with reproducible, standardized steps, that can be implemented by most corneal surgeons in any clinical settings and with relatively low costs.[71]

Compared to PK, lamellar keratoplasty procedures provide faster recovery, better visual results, with less scarring and fewer optical stromal aberrations. In addition, lower rates of post-operative endothelial cell loss have been reported in long-term outcome studies. [72]

Globally, the main barrier to patients receiving these treatments is the lack of suitable donor tissue. To cope with global shortage of donor corneas, the concept of using one donor cornea for the treatment of multiple patients was realized with the introduction of the ‘Hemi-DMEK’[73–75] and ‘Quarter-DMEK’.[76–79] These approaches use one donor cornea to prepare two semi-circular or four quadrant-shaped DMEK grafts with the potential to double or quadruple the availability of endothelial grafts. These techniques are surgically similar to standard DMEK and achieved comparable visual acuity outcomes, however, the initial case series showed higher rates of graft detachment and relatively low postoperative endothelial cell density.[80,81] Another limitation of these techniques, particularly for Quarter-DMEK, is that the surgical indication is mostly restricted to cases of Fuchs endothelial corneal dystrophy with central corneal guttata (Figure 5B) and without peripheral corneal edema on slit-lamp examination.

Clinical reports of patients who achieved corneal clearing despite failure of graft adhesion after endothelial keratoplasty[82,83] stimulated research into an entirely different concept – the possibility of cell free treatments. The initial cases with corneal clearance despite graft detachment were followed by reports of endothelial regeneration after Descemet’s Stripping Only (DSO) procedures, also known as Descemetorhexis without endothelial keratoplasty (DWEK), that has emerged as a new tissue-free treatment option for patients with central FECD.[84–86] Like any surgical technique, DSO has also evolved over time. Currently, only a small area of diseased DM with a diameter of about 4–6mm, along with its endothelial cells and guttae in the visual axis, is removed. The bare area is then left to clear by the migration of peripheral endothelial cells. The presence of healthy peripheral cells is therefore a pre-requisite for successful DSO treatment but despite improvements in high resolution imaging technologies to determine the peripheral cell density reserve, some patients still do not clear after DSO.[87] The complexity of determining not only the density but the migration capacity and quality of the peripheral endothelial cell reserve in order to define the ideal candidate for DSO, may be the major limiting factor on the widespread use of this approach[88] which shows a 12-month failure rate of 8% - 18%.[89–94] Recently, studies have reported on the topical administration of Rho-kinase inhibitors (ROCK-inhibitors) in combination with DSO.[95–96] It has been shown that the use of ROCK-inhibitors promotes the

recovery of ECD, of visual quality, and supports the concept of peripheral endothelial cell proliferation and/or migration by decreasing peripheral ECD loss. Therefore, by combining DSO with ROCK inhibitors an important economical saving for society could take place as it does not require donor tissue nor expensive post-operative care.

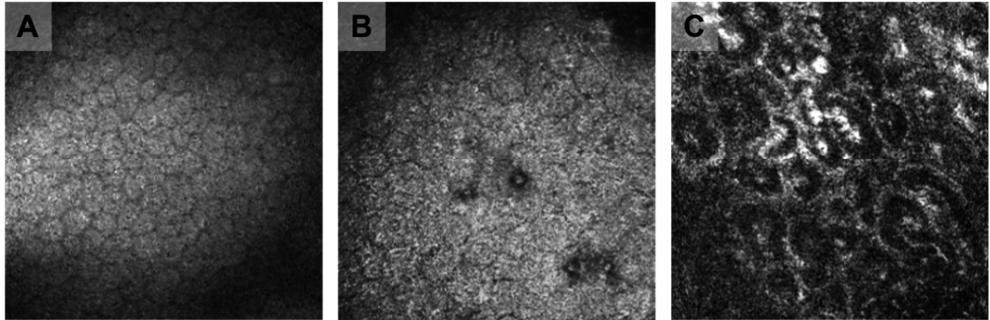


Figure 5 | *In vivo* confocal microscopy images: (A) healthy corneal endothelium (B) mild FECD: guttae appear rather scattered and appear in only a small portion of the endothelial area, and (C) severe FECD: confluent guttae visible across the entire endothelium.

DSO/DWEK is likely to only be an option for milder FECD cases and will not eliminate the demand for corneal tissue. With this in mind, other alternatives for donor graft material are being investigated. Several clinical trials are underway evaluating approaches to using corneal endothelial cell replacement therapy through the injection of cultivated cells from a donor in the presence of mitogens[97,98] or even loaded with magnetic nanoparticles.[99] Expanding cells in culture allows far more patients to be treated by a single corneal donor. The longest-running trial using cell injection delivery has enrolled more than 60 participants in Japan[100] and recently reported its 5-year follow-up of the first 11 patients.[101] The most recently reported clinical trial with cultured endothelial cells was conducted from November 2020 to May 2021 at the Quesada Clinic in San Salvador, El Salvador, and the investigators injected cultured cells of two donor corneas into 50 affected eyes with the goals of reducing corneal edema and restoring vision. Although official data is not yet available, the investigators have confidence in the procedure's efficiency.[102] Figure 6 summarizes the current and developing regenerative medicine therapies to treat corneal endothelial disease.

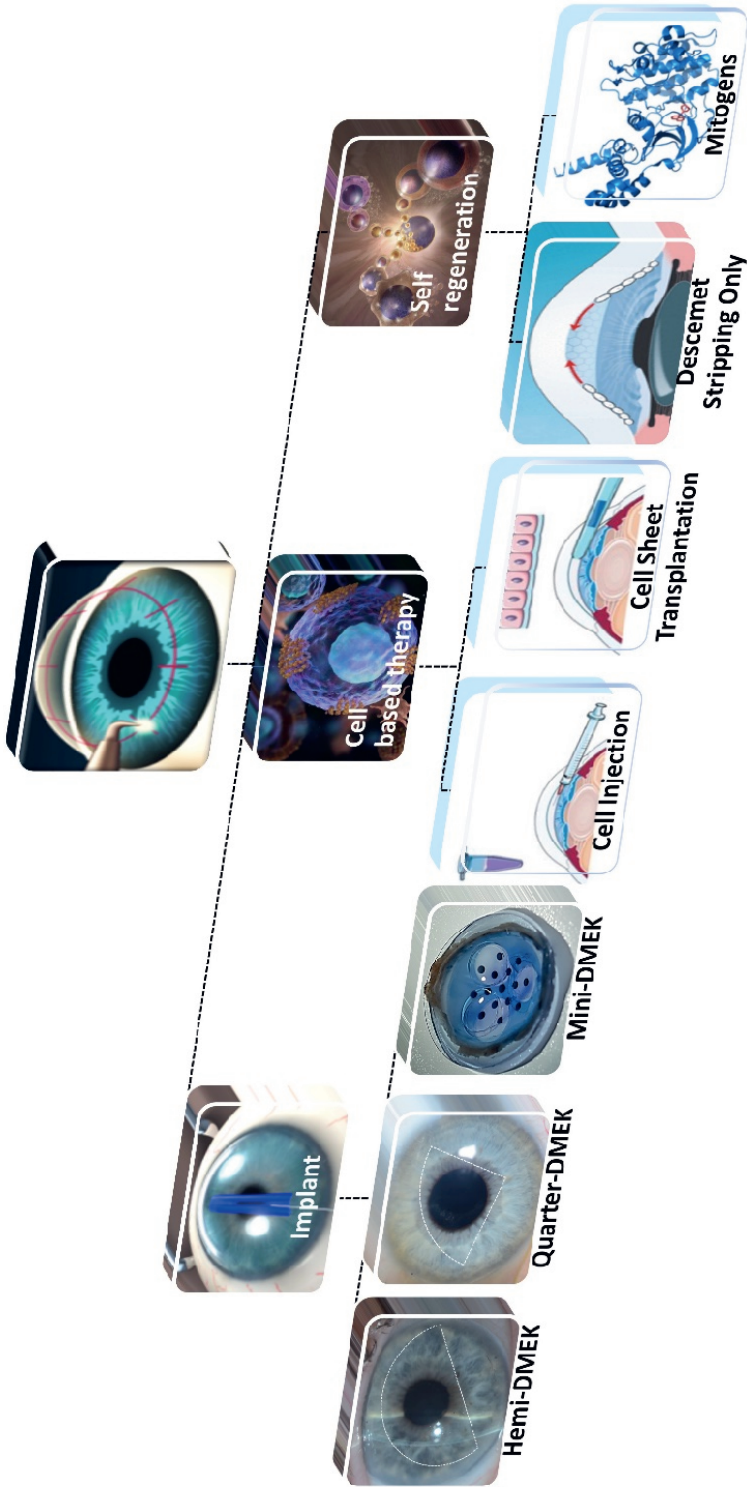


Figure 6 | Strategies to counteract the global shortage of donor material. Implant approach: efficient use of donor cornea with Hemi-DMEK, Quarter-DMEK, Mini-DMEK. Cell based therapy: cell injection using cultured cells of mature differentiated cells or cell sheet transplantation. Self regeneration through Descemet stripping only in the presence or absence of mitogens.

AIM AND THESIS OUTLINE

The thesis focuses on the *in vivo* and *in vitro* behavior of corneal endothelial cells before and after endothelial keratoplasty. The first part of the project concentrates on the ECD decrease after DMEK and DMEK graft viability prior to transplantation. The second part focusses on regenerative strategies for the treatment of FECD by developing and applying *in vitro* cell migration assays. *In vitro* cell migration from DMEK grafts of various sizes and shapes are investigated in a 3D cell culture system aiming to identify critical parameters for the successful clinical application of corneal endothelial therapies.

The first two chapters focus on the pre- and postoperative ECD measurements of DMEK grafts. In **Chapter 2**, we analyse ECD decrease after DMEK and demonstrate that about half of the observed ECD decrease at 6 months after DMEK is an *in vivo* decline from 1 day to 6 months postoperatively. The remaining decrease between preoperative and 1 day postoperative ECD values may be attributed to measurement error in the eye bank. In **Chapter 3**, we further examine the high ECD drop in the early postoperative phase after DMEK by analyzing the effect of graft preparation and organ-culture storage on ECD and viability of DMEK grafts prior to their release for transplantation.

The next six chapters focus on obtaining a better understanding of the regenerative capacity of the corneal endothelium by collecting the latest updates on the corneal endothelial wound process and performing explant outgrowth assays using a novel 3D culture technique. In **Chapter 4**, we summarize what is currently known about the wound healing characteristics from a biochemical level in the lab to the regenerative features seen in the clinic. In **Chapter 5**, cell migration of shape-adapted DMEK (Quarter-DMEK) grafts is replicated in an *in vitro* culture system and possible reasons for the lack of endothelial cell migration from the peripheral round edge of Quarter-DMEK grafts are examined. In **Chapter 6**, a novel 3D culture technique for an improved studying *in vitro* cell migration from explant tissue is tested. The technical 'ins and outs' of the proposed culture system and the cell ability to remodel the artificial matrix during migration while the explant tissue is maintained and fixed on a rigid position are analysed. In **Chapter 7**, we use the 3D culture system to explain the migration capacity of the peripheral endothelium, to provide more effective graft modifications prior to clinical implementation. In **Chapter 8**, we determine the migration of peripheral corneal endothelial cells in the presence and absence of mitogens to better understand the cell migration mechanism from phenotypically-distinct regions of the endothelium. In **Chapter 9**, a new preparation process and surgical testing of small diameter DMEK grafts are investigated. Additionally, by engaging the 3D hydrogel system, the surgical effect on endothelial cell density, viability, and migration capacity is evaluated.

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Chapter 1

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Chapter 1

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