Transplantation of cultured corneal endothelial cells: Towards clinical application

Spinozzi D.

CHAPTER 1

Introduction and outline of the thesis

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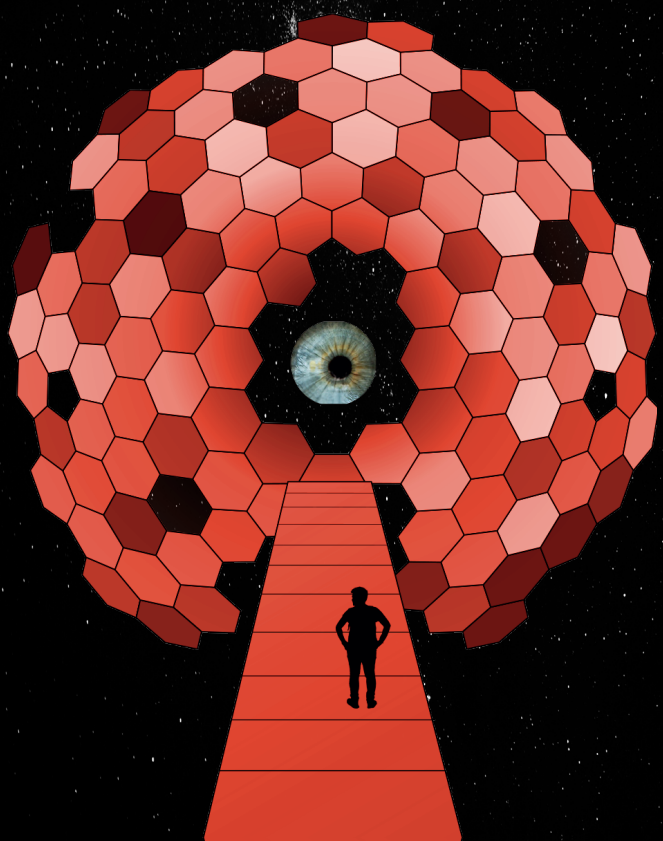

INTRODUCTION

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]

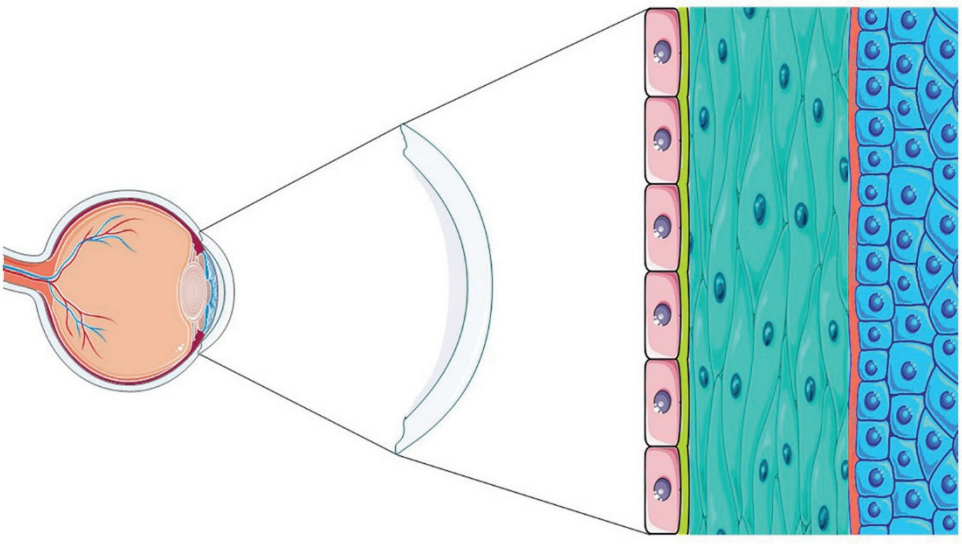

Figure 1: The human cornea. Schematic representation of the human cornea and its position within the human eye. Several layers can be distinguished: corneal endothelium (in pink, average thickness of 5 μm), Descemet's membrane (in yellow, average thickness of 10-12 μm), corneal stroma (in green, average 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]

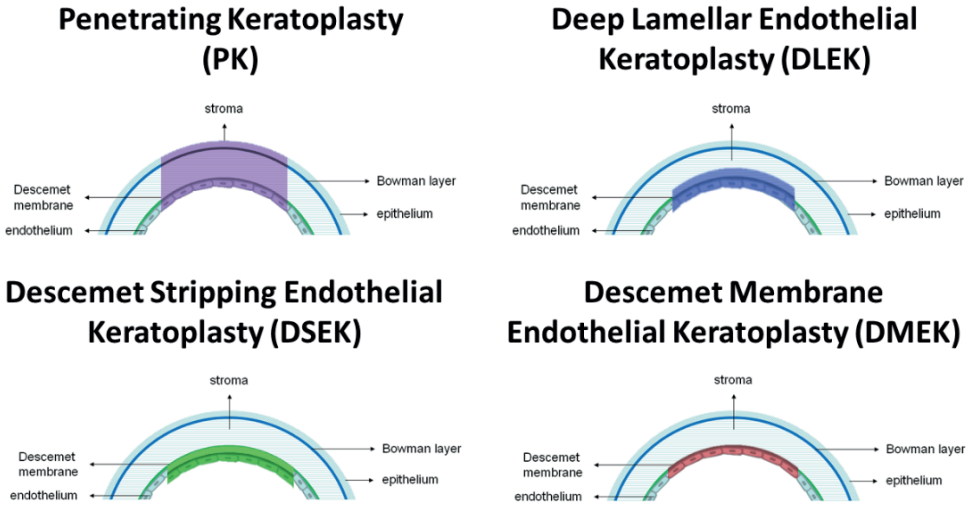

Figure 2: Development of endothelial keratoplasty. For many years, the only possibility to perform a corneal transplantation was penetrating keratoplasty (PK), a technique that required the application of sutures, with consequent very slow recovery of the patient. From the late 1990s, this surgical technique 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]

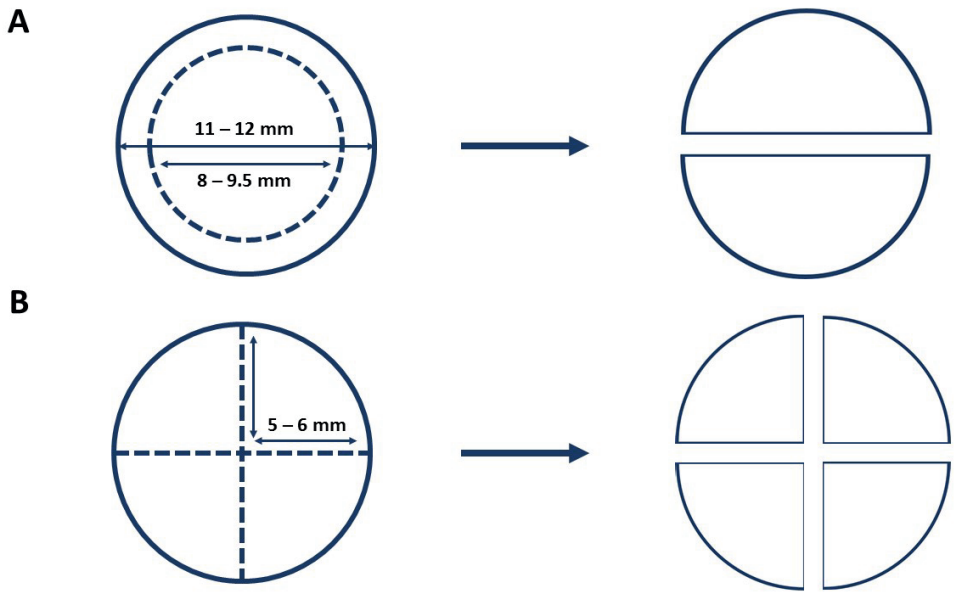

Figure 3: Hemi- and quarter-DMEK graft preparation. Schematic description of the preparation of hemi- and quarter-DMEK grafts. For hemi-DMEK grafts, a full-size endothelial graft is cut longitudinally in 2 semicircular halves with a 11-12 mm diameter, so to roughly cover the surface area of a standard 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.

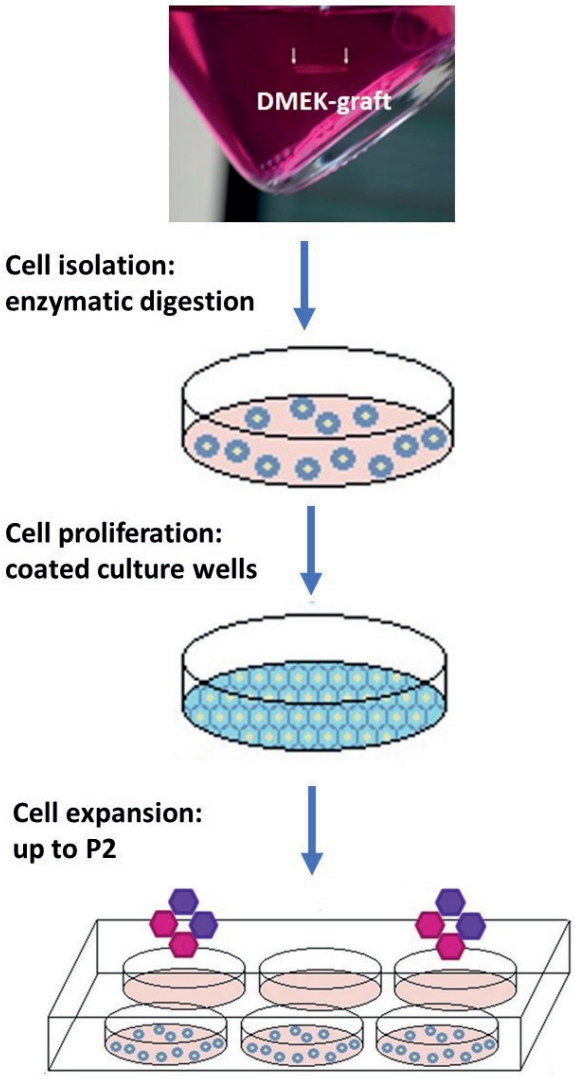

Figure 4: Human CEC isolation and culture protocol. *In vitro* hCEC culture starts from the peeling of the endothelial graft from a donor cornea. Tissue digestion allows the formation of single cells that are put in the condition to proliferate onto coated culture wells. Upon confluency, hCEC are passaged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

REFERENCES

1. Sridhar MS. Anatomy of cornea and ocular surface. Indian J Ophthalmol. 2018;66(2):190-4.
2. Srinivas SP. Dynamic regulation of barrier integrity of the corneal endothelium. Optom Vis Sci. 2010;87(4):E239-54.
3. Beuerman RW, Pedroza L. Ultrastructure of the human cornea. Microsc Res Tech. 1996;33(4):320-35.
4. Harrison TA, He Z, Boggs K, Thuret G, Liu HX, Defoe DM. Corneal endothelial cells possess an elaborate multipolar shape to maximize the basolateral to apical membrane area. Mol Vis. 2016;22:31-9.
5. He Z, Forest F, Gain P, Rageade D, Bernard A, Acquart S, et al. 3D map of the human corneal endothelial cell. Sci Rep. 2016;6:29047.
6. Kaufman HE, Capella JA, Robbins JE. The human corneal endothelium. Am J Ophthalmol. 1966;61(5 Pt 1):835-41.
7. Waring GO, 3rd, Bourne WM, Edelhauser HF, Kenyon KR. The corneal endothelium. Normal and pathologic structure and function. Ophthalmology. 1982;89(6):531-90.
8. Bourne WM. Clinical estimation of corneal endothelial pump function. Trans Am Ophthalmol Soc. 1998;96:229-39; discussion 39-42.
9. Riley MV, Winkler BS, Starnes CA, Peters MI, Dang L. Regulation of corneal endothelial barrier function by adenosine, cyclic AMP, and protein kinases. Invest Ophthalmol Vis Sci. 1998;39(11):2076-84.
10. Polse KA, Brand R, Mandell R, Vastine D, Demartini D, Flom R. Age differences in corneal hydration control. Invest Ophthalmol Vis Sci. 1989;30(3):392-9.
11. Olsen EG, Davanger M, Moen T. The role of microfilaments in the healing of the corneal endothelium. Acta Ophthalmol (Copenh). 1985;63(1):104-8.
12. Petroll WM, Hsu JK, Bean J, Cavanagh HD, Jester JV. The spatial organization of apical junctional complex-associated proteins in feline and human corneal endothelium. Curr Eye Res. 1999;18(1):10-9.
13. Barry PA, Petroll WM, Andrews PM, Cavanagh HD, Jester JV. The spatial organization of corneal endothelial cytoskeletal proteins and their relationship to the apical junctional complex. Invest Ophthalmol Vis Sci. 1995;36(6):1115-24.
14. Mohay J, McLaughlin BJ. Corneal endothelial wound repair in normal and mitotically inhibited cultures. Graefes Arch Clin Exp Ophthalmol. 1995;233(11):727-36.
15. Joyce NC, Meklir B, Joyce SJ, Zieske JD. Cell cycle protein expression and proliferative status in human corneal cells. Invest Ophthalmol Vis Sci. 1996;37(4):645-55.
16. Joyce NC, Harris DL, Mello DM. Mechanisms of mitotic inhibition in corneal endothelium: contact inhibition and TGF-beta2. Invest Ophthalmol Vis Sci. 2002;43(7):2152-9.
17. Joyce NC. Proliferative capacity of the corneal endothelium. Prog Retin Eye Res. 2003;22(3):359-89.

18. Bourne WM, Nelson LR, Hodge DO. Central corneal endothelial cell changes over a ten-year period. Invest Ophthalmol Vis Sci. 1997;38(3):779-82.
19. Gambato C, Longhin E, Catania AG, Lazzarini D, Parrozzani R, Midena E. Aging and corneal layers: an in vivo corneal confocal microscopy study. Graefes Arch Clin Exp Ophthalmol. 2015;253(2):267-75.
20. Murphy C, Alvarado J, Juster R, Maglio M. Prenatal and postnatal cellularity of the human corneal endothelium. A quantitative histologic study. Invest Ophthalmol Vis Sci. 1984;25(3):312-22.
21. Amann J, Holley GP, Lee SB, Edelhauser HF. Increased endothelial cell density in the paracentral and peripheral regions of the human cornea. Am J Ophthalmol. 2003;135(5):584-90.
22. DelMonte DW, Kim T. Anatomy and physiology of the cornea. J Cataract Refract Surg. 2011;37(3):588-98.
23. Engelmann K, Bednarz J, Valtink M. Prospects for endothelial transplantation. Exp Eye Res. 2004;78(3):573-8.
24. Schmedt T, Silva MM, Ziaei A, Jurkunas U. Molecular bases of corneal endothelial dystrophies. Exp Eye Res. 2012;95(1):24-34.
25. Duman F, Kosker M, Suri K, Reddy JC, Ma JF, Hammersmith KM, et al. Indications and outcomes of corneal transplantation in geriatric patients. Am J Ophthalmol. 2013;156(3):600-7 e2.
26. Price FW, Jr., Price MO. Evolution of endothelial keratoplasty. Cornea. 2013;32 Suppl 1:S28-32.
27. Pineros O, Cohen EJ, Rapuano CJ, Laibson PR. Long-term results after penetrating keratoplasty for Fuchs' endothelial dystrophy. Arch Ophthalmol. 1996;114(1):15-8.
28. Melles GR, Eggink FA, Lander F, Pels E, Rietveld FJ, Beekhuis WH, et al. A surgical technique for posterior lamellar keratoplasty. Cornea. 1998;17(6):618-26.
29. Terry MA. Deep lamellar endothelial keratoplasty (DLEK): pursuing the ideal goals of endothelial replacement. Eye (Lond). 2003;17(8):982-8.
30. Terry MA, Ousley PJ. Deep lamellar endothelial keratoplasty visual acuity, astigmatism, and endothelial survival in a large prospective series. Ophthalmology. 2005;112(9):1541-8.
31. Fernandez MM, Afshari NA. Endothelial Keratoplasty: From DLEK to DMEK. Middle East Afr J Ophthalmol. 2010;17(1):5-8.
32. Melles GR, Wijdh RH, Nieuwendaal CP. A technique to excise the descemet membrane from a recipient cornea (descemetorhexis). Cornea. 2004;23(3):286-8.
33. Price FW, Jr., Price MO. Descemet's stripping with endothelial keratoplasty in 50 eyes: a refractive neutral corneal transplant. J Refract Surg. 2005;21(4):339-45.
34. Gorovoy MS. Descemet-stripping automated endothelial keratoplasty. Cornea. 2006;25(8):886-9.
35. Melles GR, Ong TS, Ververs B, van der Wees J. Descemet membrane endothelial keratoplasty (DMEK). Cornea. 2006;25(8):987-90.

36. Dapena I, Moutsouris K, Droutsas K, Ham L, van Dijk K, Melles GR. Standardized "no-touch" technique for descemet membrane endothelial keratoplasty. Arch Ophthalmol. 2011;129(1):88-94.
37. Melles GR, Dapena I. How to get started with standardized 'no-touch' Descemet membrane endothelial keratoplasty (DMEK). : Netherlands Institute for Innovative Ocular Surgery; 2014.
38. Ham L, Balachandran C, Verschoor CA, van der Wees J, Melles GR. Visual rehabilitation rate after isolated descemet membrane transplantation: descemet membrane endothelial keratoplasty. Arch Ophthalmol. 2009;127(3):252-5.
39. Price MO, Giebel AW, Fairchild KM, Price FW, Jr. Descemet's membrane endothelial keratoplasty: prospective multicenter study of visual and refractive outcomes and endothelial survival. Ophthalmology. 2009;116(12):2361-8.
40. Anshu A, Price MO, Price FW, Jr. Risk of corneal transplant rejection significantly reduced with Descemet's membrane endothelial keratoplasty. Ophthalmology. 2012;119(3):536-40.
41. Builles N, Kodjikian L, Burillon C, Damour O. Major endothelial loss from corneas in organ culture: importance of second endothelial count. Cornea. 2006;25(7):815-20.
42. McColgan K. Corneal transplant surgery. J Perioper Pract. 2009;19(2):51-4.
43. Gain P, Jullienne R, He Z, Aldossary M, Acquart S, Cognasse F, et al. Global Survey of Corneal Transplantation and Eye Banking. JAMA Ophthalmol. 2016;134(2):167-73.
44. Lam FC, Baydoun L, Dirisamer M, Lie J, Dapena I, Melles GR. Hemi-Descemet membrane endothelial keratoplasty transplantation: a potential method for increasing the pool of endothelial graft tissue. JAMA Ophthalmol. 2014;132(12):1469-73.
45. Zygoura V, Baydoun L, Ham L, Bourgonje VJA, van Dijk K, Lie JT, et al. Quarter-Descemet membrane endothelial keratoplasty (Quarter-DMEK) for Fuchs endothelial corneal dystrophy: 6 months clinical outcome. Br J Ophthalmol. 2018;102(10):1425-30.
46. Garcerant D, Hirnschall N, Toalster N, Zhu M, Wen L, Moloney G. Descemet's stripping without endothelial keratoplasty. Curr Opin Ophthalmol. 2019;30(4):275-85.
47. Iovieno A, Neri A, Soldani AM, Adani C, Fontana L. Descemetorhexis Without Graft Placement for the Treatment of Fuchs Endothelial Dystrophy: Preliminary Results and Review of the Literature. Cornea. 2017;36(6):637-41.
48. Arbelaez JG, Price MO, Price FW, Jr. Long-term follow-up and complications of stripping descemet membrane without placement of graft in eyes with Fuchs endothelial dystrophy. Cornea. 2014;33(12):1295-9.
49. Borkar DS, Veldman P, Colby KA. Treatment of Fuchs Endothelial Dystrophy by Descemet Stripping Without Endothelial Keratoplasty. Cornea. 2016;35(10):1267-73.
50. Moloney G, Petsoglou C, Ball M, Kerdraon Y, Hollhumer R, Spiteri N, et al. Descemetorhexis Without Grafting for Fuchs Endothelial Dystrophy-Supplementation With Topical Ripasudil. Cornea. 2017;36(6):642-8.
51. Lie JT, Lam FC, Groeneveld-van Beek EA, van der Wees J, Melles GR. Graft preparation for hemi-Descemet membrane endothelial keratoplasty (hemi-DMEK). Br J Ophthalmol. 2016;100(3):420-4.

52. Lam FC, Baydoun L, Satue M, Dirisamer M, Ham L, Melles GR. One year outcome of hemi-Descemet membrane endothelial keratoplasty. Graefes Arch Clin Exp Ophthalmol. 2015;253(11):1955-8.
53. Birbal RS, Hsien S, Zygoura V, Parker JS, Ham L, van Dijk K, et al. Outcomes of Hemi-Descemet Membrane Endothelial Keratoplasty for Fuchs Endothelial Corneal Dystrophy. Cornea. 2018;37(7):854-8.
54. Muller TM, Lavy I, Baydoun L, Lie JT, Dapena I, Melles GR. Case Report of Quarter-Descemet Membrane Endothelial Keratoplasty for Fuchs Endothelial Dystrophy. Cornea. 2017;36(1):104-7.
55. Baydoun L, Zygoura V, Hsien S, Birbal RS, Spinozzi D, Lie JT, et al. Clinical feasibility of using multiple grafts from a single donor for Quarter-DMEK. Acta Ophthalmol. 2018;96(5):e656-e8.
56. Birbal RS, Ni Dhubhghaill S, Baydoun L, Ham L, Bourgonje VJA, Dapena I, et al. Quarter-Descemet Membrane Endothelial Keratoplasty: One- to Two-Year Clinical Outcomes. Cornea. 2020;39(3):277-82.
57. Miron A, Spinozzi D, Bruinsma M, Lie JT, Birbal RS, Baydoun L, et al. Asymmetrical endothelial cell migration from in vitro Quarter-Descemet membrane endothelial keratoplasty grafts. Acta Ophthalmol. 2018;96(8):828-33.
58. Miron A, Spinozzi D, Ni Dhubhghaill S, Lie JT, Oellerich S, Melles GRJ. In vitro endothelial cell migration from limbal edge-modified Quarter-DMEK grafts. PLoS One. 2019;14(11):e0225462.
59. Nakahara M, Okumura N, Kay EP, Hagiya M, Imagawa K, Hosoda Y, et al. Corneal endothelial expansion promoted by human bone marrow mesenchymal stem cell-derived conditioned medium. PLoS One. 2013;8(7):e69009.
60. Aboalchamat B, Engelmann K, Bohnke M, Eggli P, Bednarz J. Morphological and functional analysis of immortalized human corneal endothelial cells after transplantation. Exp Eye Res. 1999;69(5):547-53.
61. Bednarz J, Teifel M, Friedl P, Engelmann K. Immortalization of human corneal endothelial cells using electroporation protocol optimized for human corneal endothelial and human retinal pigment epithelial cells. Acta Ophthalmol Scand. 2000;78(2):130-6.
62. Joyce NC, Harris DL. Decreasing expression of the G1-phase inhibitors, p21Cip1 and p16INK4a, promotes division of corneal endothelial cells from older donors. Mol Vis. 2010;16:897-906.
63. Yokoi T, Seko Y, Yokoi T, Makino H, Hatou S, Yamada M, et al. Establishment of functioning human corneal endothelial cell line with high growth potential. PLoS One. 2012;7(1):e29677.
64. Fan T, Zhao J, Ma X, Xu X, Zhao W, Xu B. Establishment of a continuous untransfected human corneal endothelial cell line and its biocompatibility to denuded amniotic membrane. Mol Vis. 2011;17:469-80.
65. Engler C, Kelliher C, Speck CL, Jun AS. Assessment of attachment factors for primary cultured human corneal endothelial cells. Cornea. 2009;28(9):1050-4.

66. Okumura N, Ueno M, Koizumi N, Sakamoto Y, Hirata K, Hamuro J, et al. Enhancement on primate corneal endothelial cell survival in vitro by a ROCK inhibitor. Invest Ophthalmol Vis Sci. 2009;50(8):3680-7.
67. Peh GS, Beuerman RW, Colman A, Tan DT, Mehta JS. Human corneal endothelial cell expansion for corneal endothelium transplantation: an overview. Transplantation. 2011;91(8):811-9.
68. McCabe KL, Kunzevitzky NJ, Chiswell BP, Xia X, Goldberg JL, Lanza R. Efficient Generation of Human Embryonic Stem Cell-Derived Corneal Endothelial Cells by Directed Differentiation. PLoS One. 2015;10(12):e0145266.
69. Zhang K, Pang K, Wu X. Isolation and transplantation of corneal endothelial cell-like cells derived from in-vitro-differentiated human embryonic stem cells. Stem Cells Dev. 2014;23(12):1340-54.
70. Zhang C, Du L, Sun P, Shen L, Zhu J, Pang K, et al. Construction of tissue-engineered full-thickness cornea substitute using limbal epithelial cell-like and corneal endothelial cell-like cells derived from human embryonic stem cells. Biomaterials. 2017;124:180-94.
71. Chen X, Wu L, Li Z, Dong Y, Pei X, Huang Y, et al. Directed Differentiation of Human Corneal Endothelial Cells From Human Embryonic Stem Cells by Using Cell-Conditioned Culture Media. Invest Ophthalmol Vis Sci. 2018;59(7):3028-36.
72. Wu J, Izpisua Belmonte JC. Dynamic Pluripotent Stem Cell States and Their Applications. Cell Stem Cell. 2015;17(5):509-25.
73. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126(4):663-76.
74. Chen P, Chen JZ, Shao CY, Li CY, Zhang YD, Lu WJ, et al. Treatment with retinoic acid and lens epithelial cell-conditioned medium in vitro directed the differentiation of pluripotent stem cells towards corneal endothelial cell-like cells. Exp Ther Med. 2015;9(2):351-60.
75. Zhao JJ, Afshari NA. Generation of Human Corneal Endothelial Cells via In Vitro Ocular Lineage Restriction of Pluripotent Stem Cells. Invest Ophthalmol Vis Sci. 2016;57(15):6878-84.
76. Gutierrez-Aranda I, Ramos-Mejia V, Bueno C, Munoz-Lopez M, Real PJ, Macia A, et al. Human induced pluripotent stem cells develop teratoma more efficiently and faster than human embryonic stem cells regardless the site of injection. Stem Cells. 2010;28(9):1568-70.
77. Hatou S, Shimmura S. Review: corneal endothelial cell derivation methods from ES/iPS cells. Inflamm Regen. 2019;39:19.
78. Joyce NC. Proliferative capacity of corneal endothelial cells. Exp Eye Res. 2012;95(1):16-23.
79. Hayashi K, Sueishi K, Tanaka K, Inomata H. Immunohistochemical evidence of the origin of human corneal endothelial cells and keratocytes. Graefes Arch Clin Exp Ophthalmol. 1986;224(5):452-6.

80. Bohnke M, Vogelberg K, Engelmann K. Detection of neurone-specific enolase in long-term cultures of human corneal endothelium. Graefes Arch Clin Exp Ophthalmol. 1998;236(7):522-6.
81. Cvekl A, Tamm ER. Anterior eye development and ocular mesenchyme: new insights from mouse models and human diseases. Bioessays. 2004;26(4):374-86.
82. Beck RW, Gal RL, Mannis MJ, Holland EJ, Cavanagh HD, Foulks GN, et al. Is donor age an important determinant of graft survival? Cornea. 1999;18(5):503-10.
83. Miyata K, Drake J, Osakabe Y, Hosokawa Y, Hwang D, Soya K, et al. Effect of donor age on morphologic variation of cultured human corneal endothelial cells. Cornea. 2001;20(1):59-63.
84. Peh GS, Toh KP, Wu FY, Tan DT, Mehta JS. Cultivation of human corneal endothelial cells isolated from paired donor corneas. PLoS One. 2011;6(12):e28310.
85. Vernazza S, Tirendi S, Scarfi S, Passalacqua M, Oddone F, Traverso CE, et al. 2D- and 3D-cultures of human trabecular meshwork cells: A preliminary assessment of an in vitro model for glaucoma study. PLoS One. 2019;14(9):e0221942.
86. Waduthanthri KD, Montemagno C, Cetinel S. Establishment of human trabecular meshwork cell cultures using nontransplantable corneoscleral rims. Turk J Biol. 2019;43:89-98.
87. Whikehart DR, Parikh CH, Vaughn AV, Mishler K, Edelhauser HF. Evidence suggesting the existence of stem cells for the human corneal endothelium. Mol Vis. 2005;11:816-24.
88. McGowan SL, Edelhauser HF, Pfister RR, Whikehart DR. Stem cell markers in the human posterior limbus and corneal endothelium of unwounded and wounded corneas. Mol Vis. 2007;13:1984-2000.
89. Yu WY, Sheridan C, Grierson I, Mason S, Kearns V, Lo AC, et al. Progenitors for the corneal endothelium and trabecular meshwork: a potential source for personalized stem cell therapy in corneal endothelial diseases and glaucoma. J Biomed Biotechnol. 2011;2011:412743.
90. Breazzano MP, Fikhman M, Abraham JL, Barker-Griffith AE. Analysis of Schwalbe's Line (Limbal Smooth Zone) by Scanning Electron Microscopy and Optical Coherence Tomography in Human Eye Bank Eyes. J Ophthalmic Vis Res. 2013;8(1):9-16.
91. Zhang J, Ahmad AM, Ng H, Shi J, McGhee CNJ, Patel DV. Successful culture of human transition zone cells. Clin Exp Ophthalmol. 2020.
92. Kim E, Kim JJ, Hyon JY, Chung ES, Chung TY, Yi K, et al. The effects of different culture media on human corneal endothelial cells. Invest Ophthalmol Vis Sci. 2014;55(8):5099-108.
93. He Z, Campolmi N, Gain P, Ha Thi BM, Dumollard JM, Duband S, et al. Revisited microanatomy of the corneal endothelial periphery: new evidence for continuous centripetal migration of endothelial cells in humans. Stem Cells. 2012;30(11):2523-34.
94. Okumura N, Kinoshita S, Koizumi N. Cell-based approach for treatment of corneal endothelial dysfunction. Cornea. 2014;33 Suppl 11:S37-41.

95. Kinoshita S, Koizumi N, Ueno M, Okumura N, Imai K, Tanaka H, et al. Injection of Cultured Cells with a ROCK Inhibitor for Bullous Keratopathy. N Engl J Med. 2018;378(11):995-1003.