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Transplantation of cultured corneal endothelial cells: Towards clinical application

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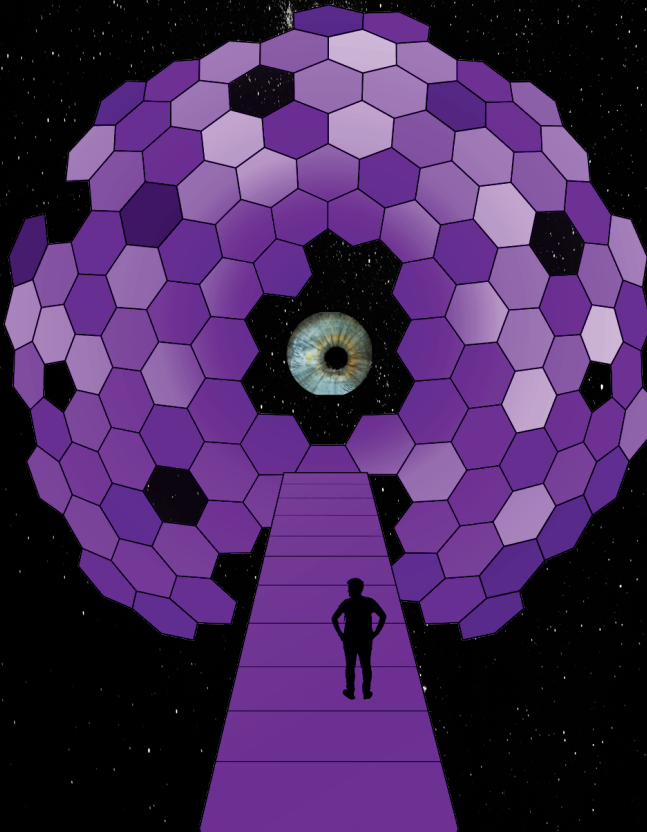
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CHAPTER 8

Discussion and Summary

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DISCUSSION AND SUMMARY

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

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

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

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

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

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

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

Optimization of the cell culture protocol for human corneal endothelial cells

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

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

In our experimental set-up, the DM-EC sheets were isolated from paired donor corneas and exposed to two different experimental conditions: (1) cell isolation immediately after DM-EC peeling and (2) cell isolation after a period of up to 6 days of storage in a growth factor-depleted medium. Our results, described in **Chapter 3**, showed that cultures established from condition #1 reported a higher success rate compared to the cultures derived from condition #2. A positive stabilization effect was also reported in terms of cell proliferation, with cultures established from condition #1 reaching cell confluency faster than the cultures derived from condition #2. Taken together, these results indicate that targeting the non-viable hCEC population before cell isolation could be an important aspect in the establishment of a reproducible *in vitro* hCEC isolation and culture protocol. Moreover, this study opens up the possibility to successfully use endothelial grafts from elderly donors for cell isolation.

Our study was conducted on single donor corneas, an important aspect to evaluate in terms of clinical application of cultured hCEC. This approach enables tissue traceability and in turn reduces the risk of rejection upon hCEC transplantation. Tissue traceability and donor-to-donor variability are more challenging to evaluate in other hCEC culture protocols, where research-grade donor tissues are pooled together before cell isolation.³²

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

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

Other research groups have outlined the importance and the feasibility to consider the research-grade corneas from elderly donors as suitable tissues for *in vitro* hCEC isolation and culture. Parekh et al. obtained hCEC cultures from old donor corneas in the presence of hyaluronic acid (HA) and Rho-kinase (ROCK) inhibitor Y-27632. Upon the evaluation of early attachment rates of hCEC on coated chamber slides and cell proliferation, they observed that isolated hCEC reached confluency within 10-15 days when cultured with HA + ROCK inhibitor.³⁵ Moreover, cultured hCEC showed a hexagonal morphology with very few cells showing polymorphism (less than 10%) and the amount of cells obtained at the end of confluency (about 2400 cells/mm²) was considered as “transplantable” for penetrating keratoplasty (PK). Another study from the same research group reported that the use of a viscoelastic solution (Viscoat) could be beneficial in the passaging of hCEC isolated from old donors. After isolation, hCEC were seeded and left to attach after a topical application of chondroitin sulphate OVD (ophthalmic viscosurgical device). This treatment led to an accelerated attachment of passaged hCEC, without loss of cell morphology and marker expression.³⁶ Moreover, more proliferative cells were seen in the cultures with OVD compared to the cultures without OVD.

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

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

Adherence of the cell culture protocol to the GMP guidelines

To allow the translation from bench to clinic of *in vitro* cultured hCEC, a suitable culture protocol has to comply with regulatory directives. The good manufacturing practice (GMP) directives provide guidelines for manufacturing, testing and quality assessment of many products, including medical devices, in order to make sure that such products are suitable and safe for human use.³⁸ GMP directives are all based on defined core principles, but they may differ depending on the country or region in which they are applied.³⁸⁻⁴²

Cell-based alternatives to corneal transplantation which are considered to be advanced therapy medicinal products (ATMP), fall under the respective regulatory directives, to provide a safe product to patients. Starting from *in vitro* hCEC culture, the use of digestion enzymes (i.e. collagenase), fetal bovine serum (FBS), growth factors to be added in culture media, and surface coatings for cell culture may provide challenges to fully comply with the GMP process, as their efficacy depends on many variables such as source and batch-to-batch variations.⁴³ Nevertheless, safe and xeno-free alternatives to the above mentioned compounds are gradually being developed and becoming commercially available, for many cell isolation protocols from tissues as well as for *in vitro* hCEC culture and expansion.^{18,44-49}

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

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

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

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

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

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

Our research focused on the feasibility of several biocompatible carriers, both bioengineered and natural, as potential alternatives to an endothelial graft for corneal endothelial transplantation. We first examined two types of substrates: GMP-produced porcine collagen scaffolds and human anterior lens capsules (HALC). Both types of carriers are already known as substrates for cell-based treatments for ocular reconstruction.^{15,52} Cultured porcine corneal endothelial cells (pCEC) were used for these tests, for several reasons: pCEC are widely used in *in vitro* studies⁵³⁻⁵⁶ and can be expanded more rapidly than cultured hCEC in order to guarantee a more efficient screening of the carrier material. As described in detail in **Chapter 5**, we showed that pCEC can be successfully cultured on collagen-based biocompatible carriers of different thickness (20 μm and 100 μm) and on HALC. Light microscopy analysis and expression of proliferation markers confirmed the ability of cultured pCEC to expand and proliferate on the abovementioned carriers. Carriers were then tested upon *in vitro* surgery in an experimental set-up composed of an artificial chamber and a human anterior remnant, namely a donor cornea without the endothelium. The pCEC-carrier constructs were tested according to some surgical parameters: rolling in BSS, staining with Trypan Blue, implantation in the artificial chamber, unfolding,

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

These results may suggest HALC to be a potential carrier for corneal endothelial cell sheet transplantation, as it has been shown that hCEC cultured on HALC retained their endothelial morphology and expressed typical markers ZO-1 and $\text{Na}^+/\text{K}^+ - \text{ATPase}$.¹⁵ HALC is a transparent membrane that protects the lens from infections and is also able to shape the lens and its surface.^{57,58} An adult HALC is composed of interacting networks of mainly laminin⁵⁹⁻⁶² and collagen type IV,^{59,63,64} while other proteoglycans, such as collagen type XVIII, collagen type XV, perlecan and fibronectin, can be also found.^{65,66} The benefits of HALC as cell carrier mostly come from its composition, that resembles that of the native DM: the core molecules self-establish a 3D matrix that gives strength, flexibility and signaling roles to the HALC,⁶⁷ although biomechanical tests show that these structural properties vary at different locations and with aging.⁶⁸ Nevertheless, using HALC does not solve the dependence from donor tissue, as one donor eye is still required for the preparation of each graft.

On the other hand, although the collagen-based carriers tested could potentially guarantee a limitless source of matrices for endothelial cell sheet transplantation, their biomechanical properties still have to be finely regulated in order to improve surgical handling and adhesion to the recipient stroma.

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

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

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

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

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

the denuded DM surface was highly structured and not smooth. This pattern, together with a likely lack of secretion of the required proteins for cell adhesion with the membrane beneath, seemed to impact the morphology of the seeded hCEC, and resulted in an impaired quality of hCEC as demonstrated also by the diffuse expression of the $\text{Na}^+/\text{K}^+ - \text{ATPase}$ marker.

The dDM might thus be a more suitable carrier, and also serve as a control for other cell-carrier constructs, if it would originate from younger donors resulting therefore in a less “imprinted” and smoother surface. However, these donors are not available at our eye bank and in most other European eye banks, and they are also not available from cataract surgery. While it would be possible on paper to purchase them from e.g. American eye banks, it would be hard to apply any obtained knowledge towards the development of tissue-engineered cell-carrier constructs, as the availability of such carriers would be even more restricted than for other types of natural carriers.

Nevertheless, after *in vivo* experiments conducted on rabbit eyes, a first-in-man clinical trial for tissue-engineered corneal grafts composed of denuded DM was recently given approval by Health Sciences Authority in Singapore (Clinical Trial Certificate: CTC1800013) for the treatment of FECD and bullous keratopathy.¹⁸

***In vivo* endothelial cell sheet transplantation in an animal model**

Before introducing the cell-carrier constructs clinically, *in vivo* studies in animal models are required, in order to identify optimal conditions for graft adhesion and maintenance of endothelial cell function *in vivo*, as well as to determine possible adverse reaction of the host to the transplanted tissue and vice versa. Primates and rabbits have been the animal models mostly used for these purposes,^{18,20,70-73} but studies can also be found with other animal models such as rats and cats.^{9,12,74-77} At the same time, pigs have been used for implantation of fish-scale biocorneas⁷⁸ and represent an interesting alternative to the aforementioned models, because of the many similarities with the biology of the human eye.

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

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

Histological examination showed a fibrotic response with associated angiogenesis caused by descemetorhexis. Interestingly, such fibrosis was milder when the endothelium was scraped, and in eyes without implantation of a pCEC-HALC construct, the scraped area included endothelial cells in number and morphology comparable to the native pig cornea. This suggests that pCEC are able to replicate *in vivo*, unlike primate CEC.⁸⁰

As a consequence the information to be obtained on the pCEC-HALC constructs from these experiments was very limited due to the surgical difficulties and the strong wound-healing reaction. Therefore, the main conclusion from these experiments was that minipigs are not a suitable animal model for testing tissue-engineered cell-carrier constructs and for endothelial keratoplasty studies in general. Nevertheless, we could show that the cell-carrier constructs can be sufficiently stained for visualization, injected onto the anterior chamber and positioned against the posterior stroma.

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

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

Future perspectives

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

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

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

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

In the last years, 3D bioprinting is rapidly becoming a promising approach for corneal replacement. Main advantages of this techniques are the high degree of customization of the corneal implants, the defined control of shape and biomechanical properties and the possibility to adopt either single- and multi-layer corneal equivalents, according to the surgical needs.⁸⁵ A combination between gene therapy and 3D bioprinting applied to hCEC has been recently described, in which cultured hCEC were transfected with a plasmid to overexpress ribonuclease 5 (R5), a protein already known for promoting cell survival in many cell types⁸⁶ including cultured hCEC.⁸⁷ R5 overexpression was a steady requirement for the following bioprinting steps, as R5-hCEC were suspended in gelatin-based bioink, and then printed on a lyophilized bovine amniotic membrane. This led to a 3D-printed construct made by 7 layers and with a thickness of 700 μm and a cell density of more than 3000 cells/ mm^2 . Upon transplantation in a rabbit model, the 3D graft started to restore the clarity of rabbit corneas, while the expression of typical corneal endothelial markers was more prominent on the 3D grafts than on the controls at 4 weeks after transplantation.⁸⁸

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

Conclusive remarks

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

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

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