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Approaches for corneal endothelium regenerative medicine

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ABSTRACT

The state of the art therapy for treating corneal endothelial disease is transplantation. Advances in the reproducibility and accessibility of surgical techniques are increasing the number of corneal transplants, thereby causing a global deficit of donor corneas and leaving 12.7 million patients with addressable visual impairment. Approaches to regenerate the corneal endothelium offer a solution to the current tissue scarcity and a treatment to those in need. Methods for generating corneal endothelial cells into numbers that could address the current tissue shortage and the possible strategies used to deliver them have now become a therapeutic reality with clinical trials taking place in Japan, Singapore and Mexico. Nevertheless, there is still a long way before such therapies are approved by regulatory bodies and become clinical practice. Moreover, acellular corneal endothelial graft equivalents and certain drugs could provide a treatment option for specific disease conditions without the need of donor tissue or cells. Finally, with the emergence of gene modulation therapies to treat corneal endothelial disease, it would be possible to treat presymptomatic patients or those presenting early symptoms, drastically reducing the need for donor tissue. It is necessary to understand the most recent developments in this rapidly evolving field to know which conditions could be treated with which approach. This article provides an overview of the current and developing regenerative medicine therapies to treat corneal endothelial disease and provides the necessary guidance and understanding towards the treatment of corneal endothelial disease.

1. Introduction

The cornea is the clear window that lets light into the eye. This avascular tissue measures 10–12 mm in diameter and 500–600 μm in thickness in adults, and has a light refractive index of 1.38 (Patel et al., 2004). The outer surface of the cornea is composed of a stratified sheet of corneal epithelial cells. The Bowman's layer, a collagen-based acellular membrane synthesized by the stromal keratocytes, separates the epithelium from the stroma. The corneal stroma accounts for 80–90% of the corneal thickness, conferring most of the tissue mechanical strength.

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It is mainly composed of highly structured collagen fibers and extracellular matrix proteins and populated by a scattered population of keratocytes, which maintain stromal homeostasis. The inner part of the cornea is lined by a monolayer of tightly packed hexagonal corneal endothelial cells (CECs) which reside in contact with the stroma on the Descemet's membrane. The adult Descemet's membrane is a 3–10 μm thick (Johnson et al., 1982) basement membrane primarily composed of collagen type IV and VIII (Kabosova et al., 2007) generated by the CECs.

CECs are thought to originate from the embryonic neural crest cells in the periocular mesenchyme. After embryonic development, human CECs are arrested at G1 phase, thus are unable to divide and lack regenerative capacity of this layer through cell division. Nevertheless, there is ongoing discussion whether a peripheral population of CECs retains some proliferative potential (Amano et al., 2006; He et al., 2012; Whikehart et al., 2005; Yam et al., 2019). Functionally, CECs act as an active metabolic pump transporting ions, namely Na^+, K^+ and Cl^-, bicarbonate, glucose, and lactic acid leading to a net basolateral/stromal to apical/aqueous humor solute flux acting as a barrier preventing the imbibition of water from the anterior chamber of the eye to the corneal stroma (Bonanno, 2003, 2012). This maintains the slightly dehydrated state of the cornea, a process called deturgescence that is fundamental to its transparency.

The average CEC density in healthy adults aged 20–39 years is 3000 cells/mm^2. This density decreases 0.3% yearly, reaching an average of 2600 cells/mm^2 in the endothelium of healthy adults aged 60–79 years (Zheng et al., 2016). Iatrogenic damage after surgery, infection or genetic diseases such as Fuchs' endothelial corneal dystrophy (FECD) can cause dysfunction and accelerated loss of CECs. Corneal endothelial disease is characterized by a loss of barrier function causing corneal edema and opacity impairing sight. Corneal opacity is one of the leading causes of blindness worldwide, and an estimated 12.7 million people worldwide are awaiting treatment (Gain et al., 2016).

2. State-of-the art: cornea transplantation

Corneal transplantation is the state-of the-art therapy for corneal endothelial disease. Since the first transplantation performed by Eduard Zirm in 1905, the cornea has become the most transplanted tissue worldwide. In 2012, 184,576 corneal transplantations were performed in 116 countries (Gain et al., 2016).

Penetrating keratoplasty effectively restores vision, but ten-year graft survival rates vary from 36 to 90% (Mannis et al., 2013; Thompson et al., 2003). The major limitations of this technique are high rates of allograft rejection and complications related to the use of sutures: astigmatism, infection, and wound dehiscence (Price et al., 2020).

Endothelial keratoplasty enables selective replacement of diseased corneal endothelium with that of a donor. Descemet stripping automated endothelial keratoplasty (DSAEK) remains the most widely used technique (Dunker et al., 2021a), but Descemet's membrane endothelial keratoplasty (DMEK) is on the rise (Dunker et al., 2021b). DMEK was first described in 2006, and allows selective replacement of the recipient's dysfunctional endothelium and Descemet's membrane (Melles et al., 2006). DMEK offers excellent and rapid recovery of vision (Dunker et al., 2020) with a low risk of allograft rejection (Birbal et al., 2020; Hos et al., 2019). However, it is technically challenging and graft detachment requiring intervention complicates about one fourth of cases (Dunker et al., 2021a).

Current research is focused on pre-loaded DMEK grafts (Fig. 1) which could be directly transported from the eye bank to the operation theatre making the procedure available for novice surgeons worldwide, reducing surgery time (Busin et al., 2018; Català et al., 2020; Newman et al., 2018; Parekh et al., 2016; Romano et al., 2018; Tran et al., 2017) and improving cost-effectiveness (Böhm et al., 2021). In an effort to overcome tissue shortage, the use of hemi (Lie et al., 2016) and even quarter DMEK (Zygoura et al., 2018) grafts has been reported. Nevertheless, given the low CEC densities reported after these procedures, increased graft detachment compared to conventional DMEK, cases of persistent peripheral corneal edema and bullae, and narrow indication for use (i.e. FECD), these techniques remain controversial and have not gained popularity. Other strategies explored to overcome tissue shortage have been the use of one donor cornea to treat two patients with different corneal pathologies, a technique also known as a split-cornea approach. The split-cornea approach optimizes the donor tissue use allowing a DMEK and a deep anterior lamellar keratoplasty to be performed with grafts originating from the same cornea (Gadhvi et al., 2020; Heindl et al., 2011).

Surgical removal of 4–5 mm of Descemet's membrane without subsequent endothelial transplantation has been described in selected cases

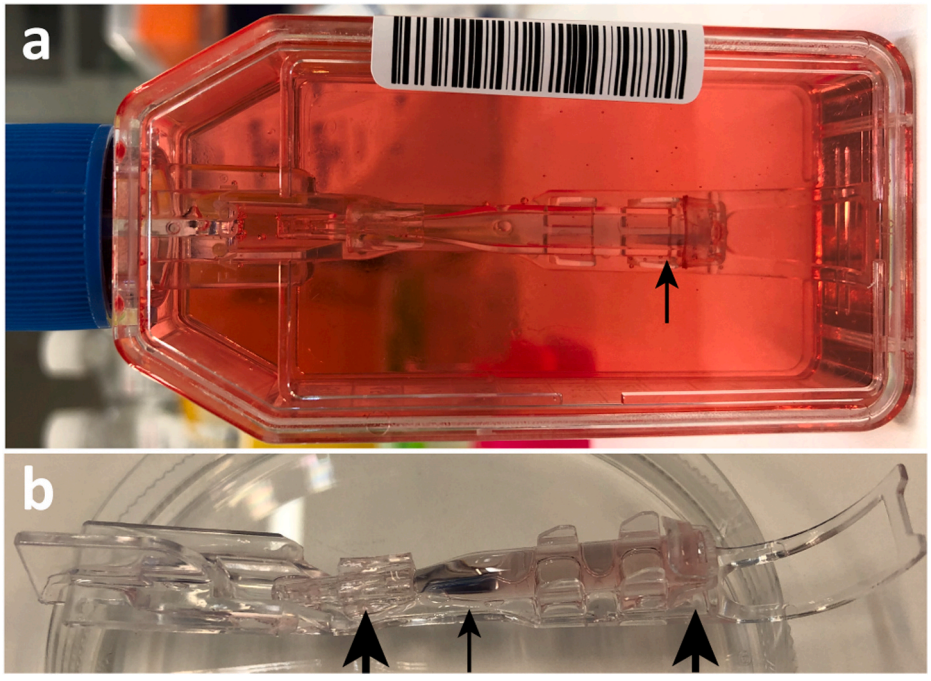


Fig. 1. Image of a DMEK graft in the preloaded DMEK cartridge system (DMEK RAPID Geuder system) in a flask containing organ culture media (A). The cartridge with transport support for the preloaded DMEK graft has two liquid permeable plugs that allow gentle washing steps and staining of the graft within the transport cartridge indicated by the arrow heads (B). Full arrows indicate the stained DMEK graft. This figure was obtained from Català et al., (2020). Licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (CC BY-NC-ND 4.0) https://creativecommons.org/licenses/by-nc-nd/4.0/.

of relatively young patients with FECD with central guttae and relatively healthy peripheral endothelium. (Arbelaez et al., 2014; Borkar et al., 2016; Shah et al., 2012). This technique known as Descemet's stripping only (DSO) or Descemetorhexis without endothelial keratoplasty (DWEK) is still in an early stage of development (Fig. 2). Current limitations are its unpredictable outcomes and a long recovery period during which the cornea remains swollen. To improve its success, this technique may require the use of pharmacological modulation with Rho-associated protein kinase (ROCK) inhibitors or the use of acellular corneal endothelial graft equivalents to promote corneal endothelial regeneration, which are further discussed in Sections *5. Acellular corneal endothelial graft substitutes* and *6. Pharmacological modulation of the corneal endothelium*.

Advances in corneal transplantation are improving its reproducibility and accessibility, leading to increasing numbers of transplantations worldwide and decreasing the threshold for intervention at earlier disease stages. Unfortunately, the increase in transplantation activity aggravates the global donor tissue shortage. It has been estimated that there is only one donor cornea available for every seventy patients in need and 12.7 million people require a corneal transplantation worldwide (Gain et al., 2016). Moreover, considering the COVID-19 pandemic, tissue requisites to be deemed acceptable for transplant have become more stringent, affecting the global corneal tissue supply (Thuret et al., 2020). While improving donor cornea logistics and attitudes to donation in different societies could partially improve the current donor shortage, in our view, one of the most appealing ways to tackle the current tissue shortage problem and to make the treatment available to those in need is to develop novel and improve ongoing approaches for corneal endothelial regeneration (Fig. 3).

3. Cell sources for corneal endothelial regenerative medicine

In order to tackle the current tissue scarcity and make therapy available for more patients the *in vitro* expansion or the *de novo* generation of CECs from pluripotent stem cells or other cell sources is needed. However, challenges remain that must be overcome. The main barriers for the *in vitro* culture of CECs, are the difficulties of forcing quiescent cells to proliferate while avoiding endothelial to mesenchymal transition (EndMT), which would lead to a cellular transdifferentiation towards a myofibroblastic phenotype causing a cellular loss of function. But also the strict selection parameters for the donor tissue suitable for primary expansion. The alternative of differentiating CECs from pluripotent stem cells or other cell sources requires the development of protocols and strict end-point parameters to assure that the final cell source resembles CECs.

3.1. Primary culture of corneal endothelial cells

Attempts to culture human CECs date back to the early 1980s. At the time, published protocols significantly differed in the method used for isolating the corneal endothelium and the culture media composition for *in vitro* expansion. The selection of the culture media focused on increasing the *in vitro* proliferation capacity of CECs with different preparations of basal media (Ham's F12, Medium 199, Dulbecco's Modified Eagle Medium or OPTI-MEM-I), growth factors (nerve growth factor, basic fibroblast growth factor or epidermal growth factor), and additives (pituitary extract, calcium chloride, ascorbic acid, insulin and sodium selenite among others). The isolation techniques varied from dissecting pieces of corneal endothelium and culturing the cells via explants (Baum et al., 1979; Tripathi and Tripathi, 1982; Yue et al., 1989), generating a single-cell suspension by scraping the endothelial surface with a curved scalpel (Fabricant et al., 1981; Tripathi and Tripathi, 1982), or treating the corneal endothelium *in situ* with a collagenase-based enzymatic cocktail to generate single-cell suspensions (Engelmann et al., 1988; Engelmann and Friedl, 1989, 1995). Explant isolation was time-consuming and difficult to reproduce because of the manual variations in the technique, drawbacks which made it difficult to implement in a therapeutic setting. Moreover, the previous isolation methods were prone to contamination with stromal fibroblasts, which were undesired as the fibroblastic population would outgrow the CEC population due to its faster rate of proliferation. Furthermore, the significant donor-to-donor variability in cause of death, age, use of drugs or ethnicity made the first steps for the validation and generation of protocols to culture primary CECs more difficult.

In 2004, research performed by Amano and colleagues paved the way for the use of primary cultured human CECs in regenerative medicine. In these experiments, it was demonstrated that primary cultured CECs isolated from corneal explants could reconstruct the corneal endothelium of *ex vivo* human corneas (Amano et al., 2005) and could reverse corneal edema in rabbits and rats (Mimura et al, 2004a, 2004b, 2005).

After the introduction of Descemet's stripping in the early 2000s, primary culture protocols evolved to adopt the peel and digest approach. In this method the corneal endothelium was first mechanically stripped from the cornea and then digested into a cell suspension using a collagenase-based enzymatic digestion. This approach both increased reproducibility and drastically reduced the risk of contamination by other corneal cell types (Chen et al., 2001; Joyce and Zhu, 2004; Li et al., 2007; Peh et al., 2011; Zhu and Joyce, 2004), a necessary improvement to protocols for generating cells for clinical use.

Forcing CECs to exit their G1 phase quiescence, and enter a proliferative state, may unwittingly induce an undesired EndMT resulting in a loss of cellular function (Roy et al., 2015). EndMT is typified by a

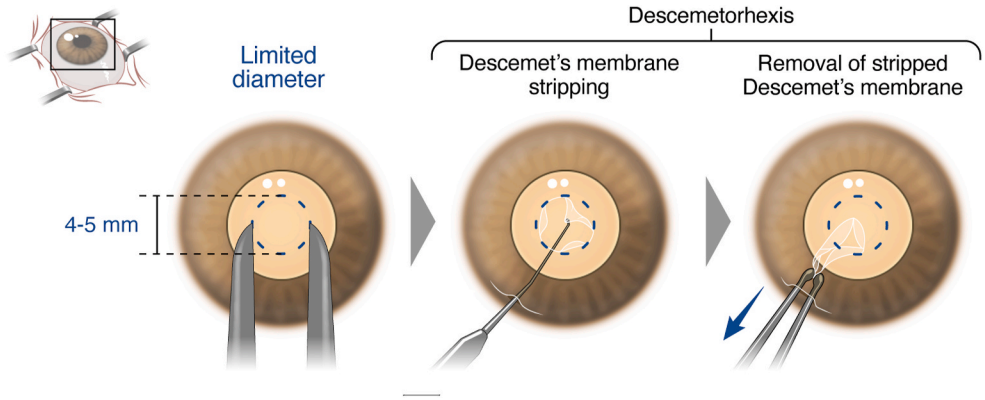


Fig. 2. Schematic representation of Descemet's stripping only (DSO). First the pupil is pharmacologically dilated for a better red reflex. Then a caliper is used to mark the central 4–5 mm diameter of the cornea. A cleavage hook is then used to fashion a small Descemet's membrane tag at the edge of the 4–5 mm mark. The tag is then grasped by forceps and Descemet's membrane is stripped.

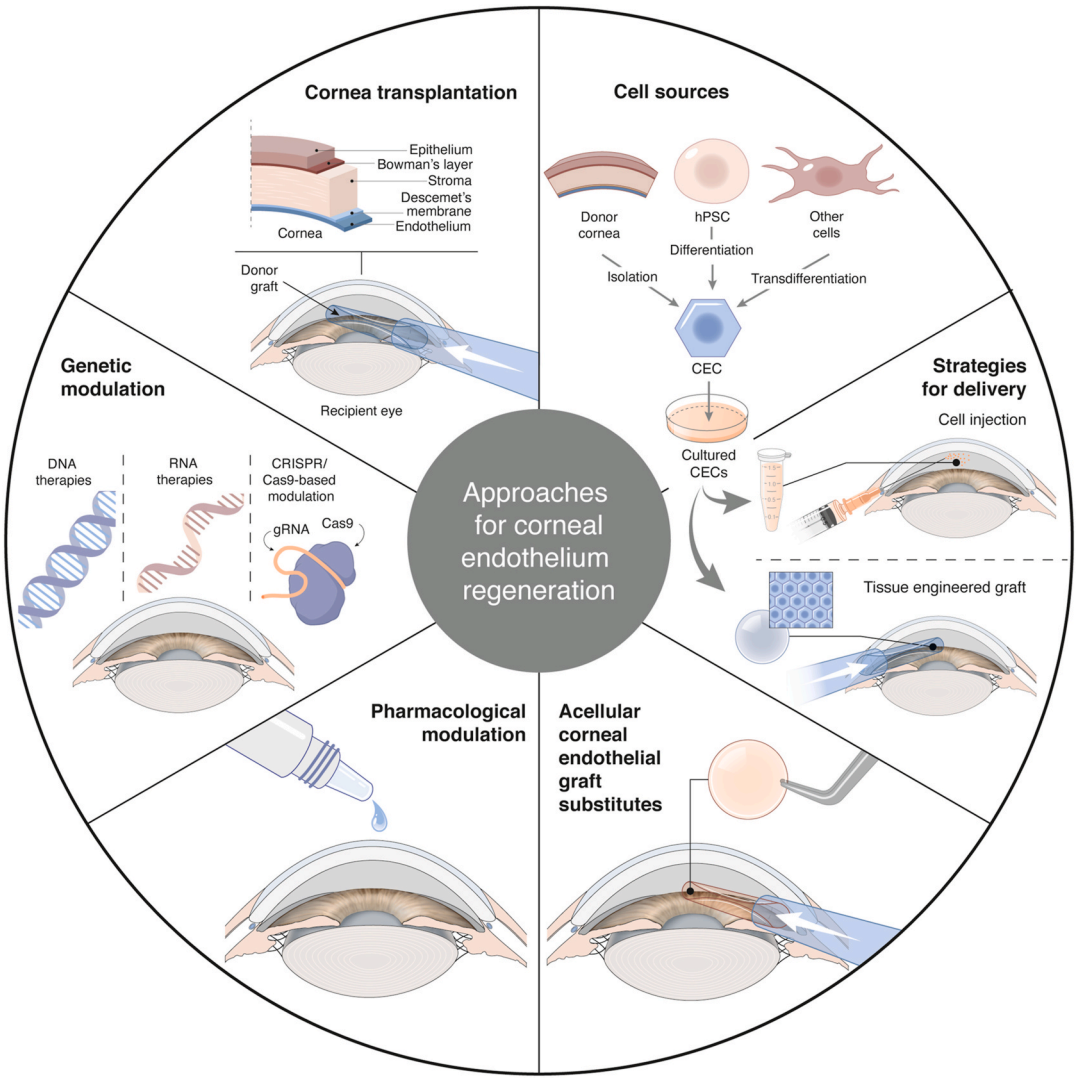


Fig. 3. There are multiple approaches for corneal endothelial regeneration that have been studied or are under development. These include cornea transplantation, cell therapies, acellular graft substitutes, pharmacological and genetic modulation of the corneal endothelium.

number of cellular events such as loss of cell–cell junctional proteins, loss of cellular polarity, reorganization of the actin cytoskeleton, increased cell mobility, abnormal extracellular matrix production, and changes to gene expression (Roy et al., 2015). EndMT is therefore one of the greatest threats in primary endothelial culture as it can render a cell product useless at best, and dangerous at worst. To date, a wide range of media and supplements have been used to culture CECs, with the main focus on promoting proliferation while maintaining the phenotype and avoiding a transition towards a mesenchymal state. By combining different basal media, fetal bovine serum and either epithelial growth factor (Joyce and Zhu, 2004; Li et al., 2007; Peh et al., 2011; Zhu and Joyce, 2004) or basic fibroblast growth factor (Engelmann and Friedl, 1995; Mimura et al, 2004a, 2004b; Peh et al., 2011), protocols have efficiently promoted *in vitro* proliferation while maintaining the cell phenotype.

Comparative studies performed in Mehta's (Peh et al., 2011) and Engelmann's (Jäckel et al., 2011) groups have shown how different media compositions affect the primary cultured CECs. In 2015, Peh and colleagues developed a unique protocol using a dual media approach to expand the corneal endothelial cells and then maintain their phenotype *in vitro* (Peh et al., 2015b). The dual media approach allowed first the expansion of the cells and then the maintenance of a confluent monolayer of corneal endothelial cells for a week using a low proliferation media and has since been widely adopted in the field (Bartakova et al., 2018; Frausto et al., 2020; Parekh et al, 2019a, 2020) (Fig. 4).

Different supplements and additives, such as pituitary extract, transferrin, ascorbic acid, calcium chloride or sodium selenite have also been studied. One of most successful approaches to increase cell proliferation and survival has been the addition of Y-27632 ROCK inhibitor, a small molecule blocker of apoptotic pathways (Kinoshita et al., 2018; Peh et al., 2015a, 2015b; Pipparelli et al., 2013). Other approaches have been the use of human serum (Vianna et al., 2015), conditioned media which increases protocol variability due to human serum incosistency (Feizi et al., 2014; Nakahara et al., 2013) and a serum-free approach (Jäckel et al., 2011), to study possible alternatives to the use of fetal bovine serum. The addition of L-ascorbate 2-phosphate (Shima et al., 2011), an antioxidant to reduce oxidative stress, and TGF-β inhibitors to avoid EndMT (Okumura et al., 2013a), have also been assessed for the primary expansion of CECs.

The improvements in the design of protocols for CEC *in vitro* expansion led to a first-in-human clinical trial. In 2018, Kinoshita and colleagues reported using primary cultured CECs to successfully restore the vision of patients with bullous keratopathy and FECD (Kinoshita et al., 2018). This landmark clinical trial succeeded in translating basic research into the clinical setting, demonstrating the therapeutic potential of primary cultured CECs.

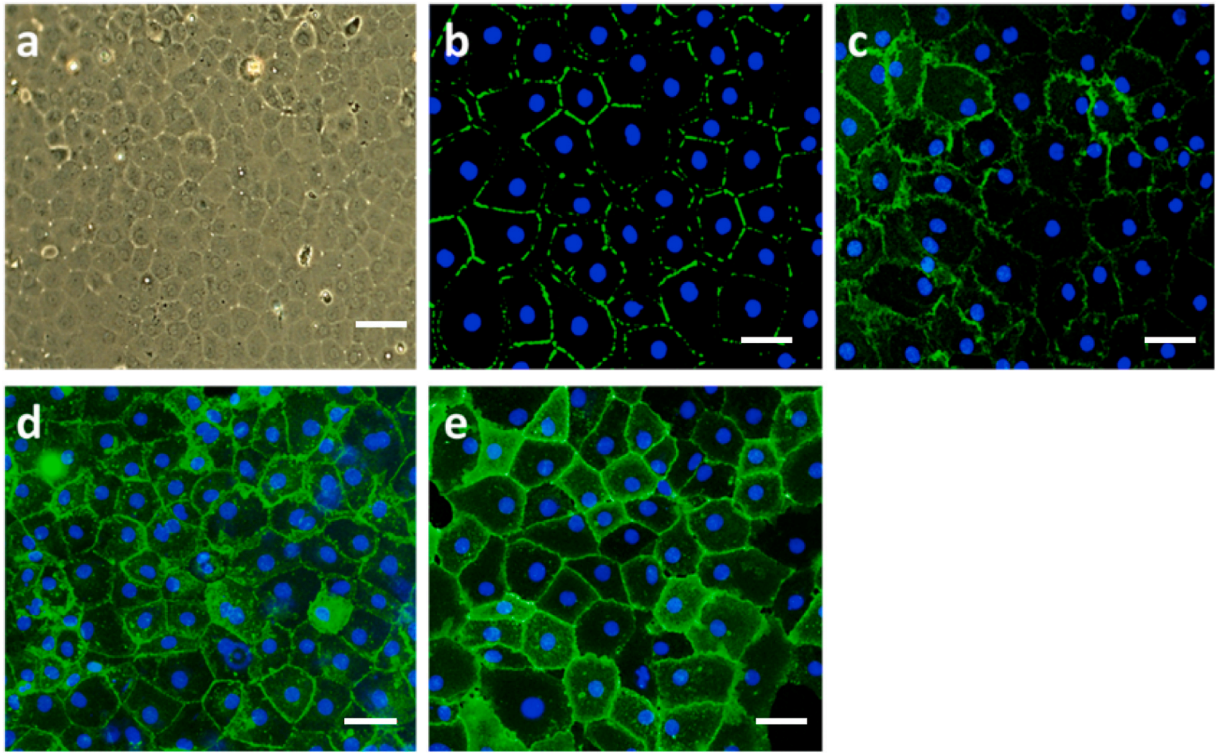


Fig. 4. Human corneal endothelial cell culture from donor tissue. Phase contrast light microscope image showing typical hexagonal cell morphology (A). Immunofluorescence analysis shows the presence of zonula occludens-1 (ZO-1) (B), Na^+/K^+ ATPase (C), CD166 (D) and Prdx6 (E) expressed by primary corneal endothelial cells. Scale bar is 100 μm (A) and 50 μm (B–E). This Figure was kindly provided by Dr Mohit Parekh.

Despite the current advances in primary culture of CECs, many questions remain unresolved. While cell seeding density (Peh et al., 2013) and young donor age (Choi et al., 2014; Miyata et al., 2001) have been directly correlated to the proliferative potential and maintenance of the CEC phenotype, these do not necessarily translate to a successful primary culture. Donor-related factors, namely the use of drugs (He et al., 2011) or oxidative stress due to high cell metabolic activity or high exposure to ultraviolet light (Joyce et al, 2009, 2011), could affect proliferation and phenotype maintenance. Sorting donors based on specific characteristics such as cause of death, age, previous pathologies, use of drugs, and other relevant factors, could be crucial to explain the varying success of *in vitro* expansion, though this requires further research and considerable numbers of research cornea. Nevertheless, developing a comprehensive and specific donor analysis could help to predict if a certain donor cornea would lead to successful *in vitro* CEC expansion and ultimately reduce waste tissue.

Another complicating factor is the method of donor tissue preservation. Most research performed to date has been done using corneas preserved in cold storage for up to 14 days. In Europe however, a warm organ culture media is usually the preferred preservation method. Most European countries cannot directly adapt the data obtained with cold storage preserved corneas to organ culture–preserved corneas. To date, there are few reports using donor corneas preserved in organ culture media (Parekh et al, 2017, 2019b2019c). The question that remains unresolved is how different storage conditions affect the expansion of primary human CECs.

Another problem to overcome is that forcing cells to exit their natural quiescence could so fundamentally change them and result in genetic and phenotypic alterations (Fig. 5). *In vitro* expansion can potentially introduce alterations in the genomic signature, affecting their phenotype with functional implications. Primary CECs can only be passaged two times before presenting genetic and functional alterations, limiting the number of cells that can be generated from a single donor cornea (Chng et al., 2013; Frausto et al, 2016, 2020). Furthermore, it is possible that during the *in vitro* expansion of CECs, different cell populations arise. Identifying the sub-populations that best resemble the native CECs

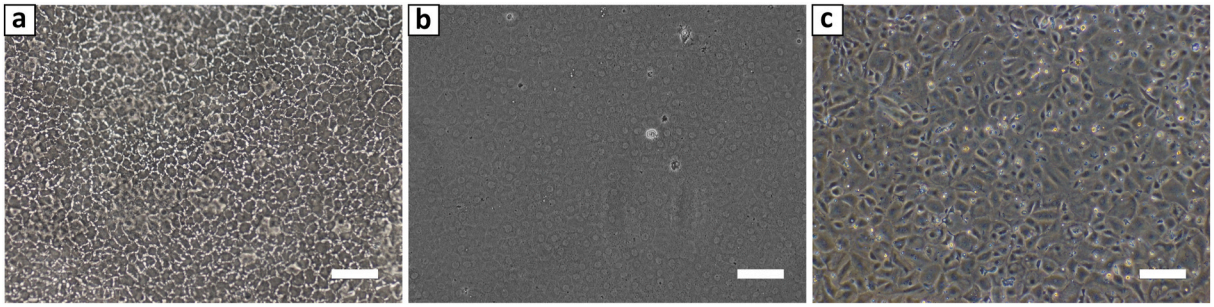


Fig. 5. Phase contrast light microscope images of human CECs in a corneal endothelium biopsy (A), primary cultured human CECs (B) and primary cultured CECs showing a characteristic morphological change experienced during primary expansion correlated with a cell loss of function and possible endothelial to mesenchymal transition (C). Scale bar is 100 μm.

based on specific markers is of utmost importance for their therapeutic application. Nevertheless, such specific markers have not been identified yet, representing another urgent area of attention.

Finally, mostly high quality corneas in terms of cell density, i.e. more than 2500 cells/mm^2, and a young age (less than 40 years) that have been used for the primary expansion of CECs. This considerably limits the number of suitable corneas, a challenge given the shortage of donors. The use of older corneas (Parekh et al, 2017, 2019b, 2019c) or discarded endothelial peripheral rims of corneas used for surgery, where cells are thought to be more proliferative (Parekh et al., 2019c, 2019d; Yam et al., 2019), would increase the availability of primary cultured cells to be used in therapy. Mehta and colleagues have recently isolated primary cells from corneas deemed unsuitable for transplantation for reasons related to connective tissue disorders, diabetes mellitus or low CEC density, to directly treat corneal bullous keratopathy in a rabbit model. This approach has the potential to increase the pool of cells available for therapy since this procedure uses non-cultured cells, and the corneas would be used for either lamellar surgery or discarded from the donor pool (Ong et al., 2020). Using alternative cell sources for CEC primary culture and regenerative medicine could drastically increase the availability of cells for therapeutic use. Nevertheless solutions for the low proliferation and rapid loss of phenotype seen with the current protocols need to be found.

3.2. Pluripotent stem cells

A new source of CECs for use in regenerative medicine could be generated from pluripotent stem cells (Fig. 6). Since Yamanaka and colleagues first introduced the concept of induced pluripotent stem cells (iPSCs) in 2006 (Takahashi and Yamanaka, 2006), stem cell–based personalized regenerative medicine has become a reality. CECs differentiated from pluripotent stem cells could be used for disease modelling and *in vitro* drug testing. Due to the low rejection index of donor tissue experienced during cornea transplantation, therapy-grade CECs could be successfully generated from both embryonic pluripotent stem cells and iPSCs. Nevertheless, such hypothesis still needs confirmation. When generating CECs from iPSCs from the patient, this risk of rejection could be further reduced. Moreover, current international initiatives to establish homozygous HLA iPSC banks will allow to overcome the logistical and financial difficulties of derivating iPSC from every single donor (Taylor et al., 2012). Overall, differentiating human pluripotent stem cells to CECs presents several advantages, such as the faster *in vitro* expansion of pluripotent stem cells compared to primary cultured CECs and independence from donor corneas. However, protocols for deriving CECs from pluripotent stem cells are still at an early developmental stage.

An intuitive way of designing a protocol for differentiating pluripotent stem cells, whether embryonic or iPSC, into CECs is to follow an approach inspired by developmental biology. CECs derive from neural crest during embryonic development (Lwigale, 2015) and most protocols published to date differentiate pluripotent stem cells into CEC–-like cells following initial neural crest induction (Ali et al., 2018; P. Chen et al., 2015; Fukuta et al., 2014; Grönroos et al., 2021; Hatou et al., 2013; Ju et al., 2012; Lovatt et al., 2018; McCabe et al., 2015; Song et al., 2016; Wagoner et al., 2018; Zhao and Afshari, 2016). The approaches used to generate a neural crest–like population from human pluripotent stem cells focus on the inhibition of the SMAD signaling pathway using the ALK5/TGF-β type I receptor kinase inhibitor SB431542 combined with either the bone morphogenetic protein antagonist Noggin (Ali et al., 2018; McCabe et al., 2015; Song et al., 2016), or the Wnt pathway regulators IWP2 and CHIR99021 (Fukuta et al., 2014; Grönroos et al., 2021; Lovatt et al., 2018; Wagoner et al., 2018). Interestingly, the use of dual SMAD inhibition with SB431542 and Noggin does not seem an intuitive method to induce a neural crest–like state as it is generally considered to be a neuroectoderm induction method (Chambers et al., 2009; Kriks et al., 2011; Pasca et al., 2015). Once the neural crest–like stage has been achieved, several approaches have been followed to induce the CEC fate. First, exposing the cells to platelet-derived growth factor-B (PDGF-B), Dickkopf-related protein 2 (DKK2) and basic fibroblast growth factor results in the generation of confluent hexagonal cells

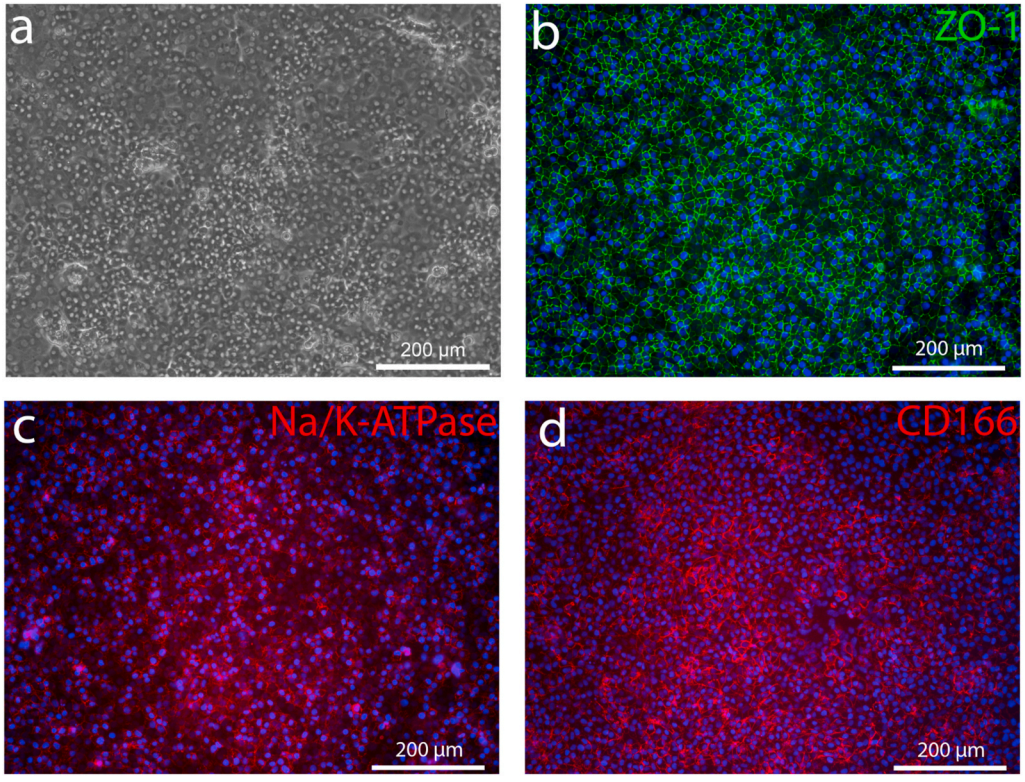


Fig. 6. Characterization of human pluripotent stem cell derived CECs. Phase contrast light microscope image shows typical hexagonal cell morphology (A). Immunofluorescence analysis shows the presence of commonly used CEC markers ZO-1 (B), Na^+/K^+ ATPase (C) and CD166 (D) expressed by human embryonic stem cell derived CECs. Representative data conducted with Regea08/017 human embryonic stem cell line. Scale bar is 200 µm. This Figure was kindly provided by Pyry Grönroos from Professor Heli Skottman's Lab.

with a CEC–like phenotype (Ali et al., 2018; McCabe et al., 2015; Song et al., 2016; Wagoner et al., 2018). A combination of SB431542 with the ROCK inhibitor H-1125 can also generate CEC–like cells (Zhao and Afshari, 2016). Moreover, a study from Skottman and colleagues portrayed the importance of retinoic acid for further differentiating neural crest like cells to CEC–like cells (Grönroos et al., 2021). In addition, the use of a recombinant laminin coating, instead of the animal–derived matrigel, could reduce the undesired batch-to-batch variability and allow a xenogeneic–free culture (Grönroos et al., 2021).

The use of primary cell conditioned media has also shown success in differentiating neural–crest like cells to CEC–like cells (Fukuta et al., 2014; Song et al., 2016). A similar differentiation approach has been used for differentiating rodent pluripotent stem cells into CECs through the neural crest–like stage (Chen et al., 2015; Hatou et al., 2013; Ju et al., 2012).

Although the developmental biology approach seems intuitive, competing approaches for inducing a direct differentiation without passing through the neural crest precursor stage are also being studied (Chen et al., 2018; Hanson et al., 2017; Li et al., 2019; Zhang et al., 2014). Direct differentiation of pluripotent stem cells into CEC–like cells has been reported by either using primary cell culture–conditioned media (Chen et al., 2018; Zhang et al., 2014), triggering spontaneous differentiation by cell seeding in corneoscleral disks (Hanson et al., 2017), or the use of a defined media containing cholera toxin, epithelial growth factor and the ROCK inhibitor Y-27632 (Li et al., 2019).

In recent years, significant progress has been made thanks to dedicated efforts to develop protocols for differentiating human pluripotent stem cells into CECs; nevertheless, there remain unsettled challenges. One of the biggest challenges is cell purity. Given the potential of these cells, undesired side populations may arise during differentiation, and these often vary between differentiation batches because of the low efficacy of current protocols. Since differentiation protocols are highly complex, characterization should be performed using an array of markers for every stage of differentiation. For early neural crest or periocular mesenchyme identification p75 (Menendez et al., 2013) or Pitx2 (Kumar and Duester, 2010) should be detected. Finally, the recent identification of CEC markers such as CD166 and sPrdx6 (Ding et al., 2014), comparable to ΔNp63α or keratin 12 in corneal epithelial cells, could open the possibility to enrich for cell populations expressing these markers and improve current differentiation protocols.

In addition to purity, there are a number of other challenges to overcome. It is crucial to demonstrate that the differentiated cells are functional and safe, and therefore suitable for therapeutic use. Functional characterization is needed to confirm the active metabolic pump activity. It is also not fully understood how the differentiation protocols affect the (epi)genomic signature of the cells and whether they induce DNA alterations in the cells such as epigenetic modifications and karyotype abnormalities. Finally, it is crucial to prove that differentiation is complete and the generated cells do not maintain any stem cell–associated pluripotency, which might lead to tumorigenic potential. Scientists aiming to bring pluripotent stem cell–derived CECs to therapy should put special focus on investigating and resolving the aforementioned matters.

3.3. Other cell sources

The differentiation of pluripotent stem cells into CECs is considered a promising method for generating a therapeutic cell source for regenerative medicine. There is growing and encouraging evidence that tumor generation will not present a prohibitive risk for therapy, but this concern still requires careful consideration. Moreover, difficulties to generate pure populations of pluripotent stem cell–derived CECs makes it difficult to implement them in a therapeutic setting. Together, these are reasons to consider other cell sources.

Transdifferentiation is a method for rapidly and reproducibly generating CECs with therapeutic potential without the associated risk and difficulties of pluripotent stem cell differentiation. It involves the reprogramming of mature somatic cells into cells of a different mature somatic lineage. Various cell types have been transdifferentiated to CEC–like cells capable of reverting corneal edema in rabbit animal models. These include bone marrow derived endothelial precursors (Shao et al., 2011), neural crest cells (Ju et al., 2012), and corneal stromal stem cells (Hatou et al., 2013). Interestingly, skin derived precursors (Inagaki et al., 2017; Shen et al., 2017) and mesenchymal stem cells (Yamashita et al., 2018) have also been transdifferentiated to CECs in a process resembling a reversed endothelial to mesenchymal transition. In order to implement such approaches in a therapeutic setting it is crucial to demonstrate the stability of transdifferentiation to assure a safe therapy and avoid a return of the cells to their somatic origin improve the transidfferentiation efficiency and purity of the existing protocols.

Taking a different approach, Joyce and colleagues used human mesenchymal stem cells to heal the damaged endothelium in human corneas *ex vivo* (Joyce et al., 2012). They showed that human mesenchymal stem cells have the capacity to adhere and repopulate denuded areas in the corneal endothelium, and possibly providing paracrine support to surrounding CECs, recovering the corneal endothelial barrier. Despite the successful preliminary results, further studies will be required in order to identify the interactions of these cells with their environment and how their genetic and phenotypic signature correlates with the native corneal endothelium.

3.4. Need for standardization of endpoint parameters

Whatever the approach for generating cells for regenerative medicine, whether primary expansion from donor cells, derivation from pluripotent stem cells, or the use of other cell sources, it is crucial to reach a consensus on endpoint parameters to assess their quality. In this section we present a perspective on the assessment criteria that generated CECs should fulfill to be used for therapeutic purposes.

3.4.1. Morphology

A parameter that can be readily assessed is the morphology of the generated CECs. A cellular hexagonal morphology upon reaching confluence in culture could be assessed to preclude the presence of spindle-shaped fibroblastic morphologies associated with a mesenchymal transition. Rating the circularity index of the generated cells confirming the hexagonality and a low polymorphism is a quality check that should be performed (Parekh et al, 2017, 2019a; Peh et al., 2015a; Peh et al., 2013). In addition to that, Yamamoto and colleagues have been able to correlate the physical intercellular interactions in a bi-dimensional *in vitro* system with a better regeneration of the corneal endothelium after cell injection (Yamamoto et al., 2019). This could be used as a tool to correlate a physical marker with the suitability of the generated cells for therapeutic use.

3.4.2. Genotype and phenotype

For their use in regenerative medicine CECs should possess a gene and protein expression comparable to native human CECs. As previously reviewed by Ni Dhubhghaill and colleagues, the most frequently used markers to characterize the generated CECs are Na^+/K^+ ATPase (*ATP1A1*), ZO-1 (*TJP1*) and collagen type VIII (*COL8*) (Van den Bogerd

et al., 2019). While each are important markers to establish the presence of metabolically active transporters, extracellular matrix production and tight junctions, respectively, these markers are not specifically expressed in the corneal endothelium but also in many other cell types such as lung (Bai et al., 2021) or intestinal epithelium (Sugi et al., 2001), and even in corneal epithelial cells (He et al., 2016b). The hexagonal phenotype is not even exclusive to the CECs in the eye; it is also prominent in the retinal pigment epithelium. Therefore, for better characterization of the generated cells, it is of utmost necessity to prove the expression of specific markers for corneal endothelium. CD166 (Ding et al., 2014; Dorfmueller et al., 2016; Okumura et al., 2014a) and sPrdx6 (Ding et al., 2014) (Fig. 4) have been recently identified as CEC markers within the cornea that correlate to therapeutic success. Moreover, a recent study by Thuret and colleagues suggested that the hexagonal shape of the CEC apical surface and the interdigitated shape of the CEC basal site together with the expression of functional and structural proteins such as CD56, CD166, Vimentin, N-cadherin and integrin a3b1 is an important hallmark of human CECs (He et al., 2016b).

It is also important to show the absence of fibroblastic markers associated with an EndMT or contamination by stromal fibroblasts, namely CD44 or CD73 (Okumura et al., 2014a). To conclude that the generated CECs are of sufficient quality to use in regenerative medicine, the characterization should thus be done by assessing a panel of diverse markers. An example of this is the panel developed and used by Kinoshita and colleagues (Kinoshita et al., 2018; Toda et al., 2017; Ueno et al., 2016). They reported that $CD166^{+}CD24^{-}CD26^{-}CD44^{-}CD105^{-}CD133^{-}$ cells have the correct gene expression and phenotype to be used in therapy. In this panel, CD166 was used as a marker for CECs and the negative makers were analyzed to exclude the fibroblastic-like phenotype (Kinoshita et al., 2018; Toda et al., 2017; Ueno et al., 2016).

3.4.3. Karyotype

During the expansion of primary CECs, they are stimulated to exit their arrested phase to proliferate. This has the potential to induce karyotype abnormalities in their genome (Hamuro et al., 2016; Miyai et al., 2008). Likewise, the generation and culture of iPSCs can also cause karyotype abnormalities (Taapken et al., 2011) and less commonly aneuploidy (Garcia-Martinez et al., 2016; Peterson et al., 2011). To prove that the cells are safe to use in regenerative medicine, it is crucial to confirm that they retain a normal and safe karyotype after their manipulation.

Kinoshita and colleagues set the basis for a clinical trial using primary expanded CECs and monitored the cell karyotype during primary expansion, nevertheless no endpoint parameter was established on what would be a suitable karyotype for therapeutic use (Kinoshita et al., 2018). In Singapore, a batch of cultured CECs is deemed unsuitable for therapy if there is a clonal chromosomal addition or deletion, such as more than two metaphase cells showing the same chromosomal trisomy, or more than three cells showing the same monosomic abnormality, or there is the presence of more than 20 cells in the metaphasic phase (Ting et al., 2021).

3.4.4. Functionality

In order to maintain corneal deturgescence and transparency, it is necessary to demonstrate that the generated cells possess their active metabolic pump activity. The expression of transporters, namely Na^{+}/K^{+} ATPase or the electrogenic sodium bicarbonate cotransporter 1 *(SLC4A4)*, is insufficient proof of functionality alone, as protein expression could not necessarily correlate to an active metabolic pump activity in the cells. To date, *in vitro*, *ex vivo* and *in vivo* methods have been developed to test the functionality of CECs.

A rapid way of demonstrating functionality is an *in vitro* test designed to show active metabolic substance transport, namely ion transport, across a monolayer of CECs. The most commonly used methods are transepithelial electrical resistance (TEER) measurements (Frausto et al., 2020) and Ussing's chamber measurements (Amano et al., 2005; Hatou et al., 2013; Mimura et al., 2004b; Zhang et al., 2014). While the first does not strictly measure transport, the sensitivity to external factors (electrode distance to measurement membrane, cell monolayer sensitivity to temperature, pH changes and cell shedding plus sensitivity of the instrumentation to vibration oscillations) of the latter makes it a difficult technique to implement.

Another possibility to assess the functionality of the CECs is to assess their active repair in *ex vivo* corneas. Maintaining the *ex vivo* corneas in a setting that mimics physiological conditions allows the measurement of corneal thickness and its correlation to cell functionality (Aboalchamat et al., 1999; Rolev et al., 2018). To fully mimic the human physiological conditions in an *ex vivo* cornea, Thuret and colleagues developed a bioreactor that opens the possibility to use *ex vivo* corneas for functionality testing (Fig. 7) (Guindolet et al., 2017).

Finally, animal models of corneal edema have also been used to test cellular functionality by measuring the decrease in the induced corneal edema (Bostan et al., 2016; Faye et al., 2021; Koizumi et al., 2012; Rolev et al., 2019). Depending on the selected model and due to interspecies physiological differences, it is crucial to perform the required controls. For instance, while rabbits are one of the most frequently used animal models, they possess a self-healing corneal endothelium. Animal models represent a valuable method to check safety and efficacy during research and development of cellular therapies (Faye et al., 2021). Nevertheless, animal models used are only representative for bullous keratopathy and there is a lack of accurate FECD models. Finally, it is not ethically justifiable to routinely use animal models as quality control to test each single batch of generated cells before clinical use.

Demonstrating CEC functionality is arguably the most important prerequisite for a successful regenerative medicinal product. Although there is no perfect test, it can be accurately assessed using a combination of methods. Nevertheless, there remains a need to develop straightforward functional testing platforms to be used in quality control before the use of each CEC batch for therapy. Organ-on-a-chip technology (Zhang et al., 2018) consists of microfluidic cell culture chips that can successfully mimic physiological responses of organs. Such a system is an interesting candidate to develop a high-throughput functional model of the corneal endothelial barrier to be systematically used as a quality control check for every batch of generated CECs.

4. Strategies for delivery of corneal endothelial cells

Advances in primary culture of CECs, pluripotent stem cell differentiation and generation of CECs from other cell sources are highly promising approaches for developing a cellular therapy to treat corneal endothelial disease. However, their success hinges on a suitable method to deliver them into the cornea (Fig. 8). Cells must be delivered alive and with sufficient potential to adhere to the posterior part of the cornea. The main two methods currently studied for delivery of CECs are cell injection into the anterior chamber of the eye and the use of different substrates in the effort of bioengineering corneal endothelial grafts (Faye et al., 2021).

4.1. Cell injection

Cell injection is the delivery of CECs in a simple and minimally invasive manner via injection in the anterior chamber of the eye (Fig. 9). In the early 2000s, Mimura and colleagues set the basis for the delivery of primary cultured CECs via intracameral injection in a rabbit bullous keratopathy model (Mimura et al, 2003, 2005). After this proof-of-concept work, research proceeded in optimizing the technique of CEC delivery via cell injection.

Gravity, for example, has been shown to increase CEC adherence to the posterior part of the cornea. After cell injection, subjects must stay in prone position for two to 3 h to enable the attachment of CECs (Mimura et al., 2007; Okumura et al., 2012). Co-delivery of the cells with the ROCK inhibitor Y-27632 combined with prone position of the recipient

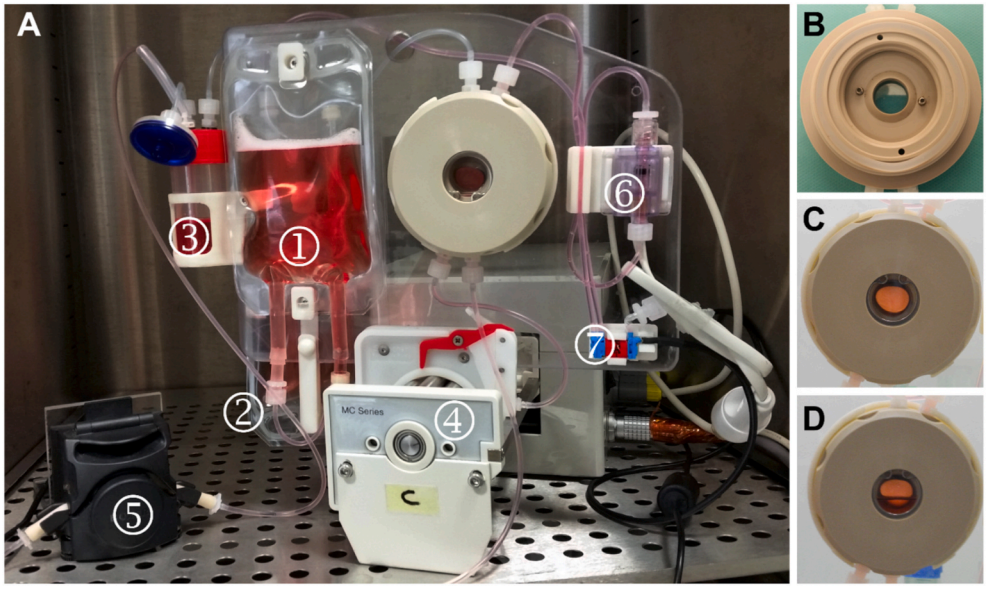


Fig. 7. Bioreactor used for the preservation of corneas in physiological-like conditions. The general set up of the bioreactor system inside a CO_2 culture incubator (A) includes a bag with fresh media for the corneal endothelial side (1), a medium waste bag (2), a flask with fresh media for the corneal epithelial side (3), two peristaltic pumps (4 and 5), a pressure sensor (6), and a miniature solenoid valve (7). (B) Empty inside of the bioreactor chamber. Bioreactor corneal chamber containing a porcine cornea after sealing the chamber (C) and during corneal medium immersion phase during operation (D). This Figure was obtained from Guindolet et al. (2017). Licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (CC BY-NC-ND 4.0) https://creativecommons.org/licenses/by-nc-nd/4.0/.

significantly improved cell adhesion (Okumura et al, 2012, 2014b; Peh et al., 2019). Another strategy explored the enhancement of CEC attachment by the use of magnetic force using cells laden with ferromagnetic beads (Mimura et al., 2003; Moysidis et al., 2015; Patel et al., 2009).

In the pioneering first-in-human clinical trial using primary cultured CECs, Kinoshita and colleagues reversed corneal edema in cataract surgery–derived bullous keratopathy patients and FECD patients with an injection of primary cultured CECs together with Y-27632 (Kinoshita et al., 2018). The landmark clinical trial from Kinoshita and colleagues has been a major milestone in the development of a cell therapy for treating corneal endothelial disease, and has promoted the identification of the aspects that need to be addressed to ensure an efficient and safe therapy. In the recent 5-year follow up study, the clinical reversal in the endothelial disease was retained in 10 of the 11 patients (Numa et al., 2020). This landmark clinical trial raised several questions. Interestingly, two different protocols for CEC primary expansion, with or without transforming growth factor β inhibitor SB431542, and two different techniques for removing damaged corneal endothelium were used (Van Den Bogerd et al., 2018). Moreover, one recipient received an injection of 5×10^5 cells while the other recipients received a cell injection of 1×10^6 cells introducing another variable factor during the clinical trial. The recipients' CEC densities 24 weeks after injection ranged from 947 to 2833 cells/mm^2 with an average density of 1924 cells/mm^2 (Kinoshita et al., 2018) which decreased to an average CEC density of 1257 cells/mm^2 after 5 years (Numa et al., 2020). It would be interesting to understand how donor and patient characteristics could influence this parameter and if the use of postoperative ROCK inhibitors could reduce the loss of CECs after their injection.

In cases of advanced FECD, where the Descemet's membrane is altered by the presence of guttae, the CEC attachment and monolayer formation is highly impaired (Kocaba et al., 2018; Rizwan et al., 2016). This can induce complications when treating these subjects with cell injection. In the clinical trial by Kinoshita and colleagues, seven patients with FECD were treated, and the guttae did not seem to improve after 2 and 5 years.

Okumura and colleagues performed a proof-of-concept study where they compared the outcomes of CEC injection in two rabbit model groups: in the first group the CECs were scraped leaving the Descemet's membrane intact, and in the second group, a 4 mm diameter Descemetorhexis was performed (Okumura et al., 2018). After 14 days, corneal thickness and transparency in both groups was comparable, although recovery in the descemetorhexis group was slower (Okumura et al., 2018). In a recent study by Mehta and colleagues, CEC injection was also performed in two rabbit models: in the first group the CECs were scraped leaving the Descemet's membrane intact, whereas in the second group a complete Descemetorhexis was performed (Peh et al., 2019). Interestingly, after three weeks, corneas in the complete Descemetorhexis group that received a CEC injection remained swollen with an approximate thickness of 850 μm, whereas in the group where Descemet's membrane was left intact, and received a CEC injection, decreased to 582 μm (Peh et al., 2019). These studies suggest that the presence of Descemet's membrane in the recipient cornea is crucial for a successful outcome of CEC injection. Nevertheless, partially removing altered parts of Descemet's membrane in a controlled manner followed by a CEC injection could be an option for treating FECD.

Cell injection is not yet an efficient method regarding the number of cells used. Namely, the number of CECs commonly used, 1×10^6 cells per cornea, is approximately 4–5 times higher than the cell count in the healthy human corneal endothelium. Based on our calculations, CECs isolated from a single donor can be expanded to 5×10^6 to 10×10^6 cells at confluence by the second passage. Adopting the cell injection numbers from Kinoshita's clinical trial, 1×10^6 cells per cornea, 5–10 patients could be hypothetically treated with one single donor. Improving cell adherence and survival during this procedure would reduce the number of cells needed to treat one diseased cornea, allowing more patients to benefit from this technique. This can be complemented by strategies such as the isolation of CECs from tissues deemed unsuitable for transplant for direct cell injection (Ong et al., 2020, *Section 3.1. Primary culture of corneal endothelial cells*).

Furthermore, it is necessary to understand how non-adhered CECs distribute. To date, there are few studies showing the biodistribution of injected CECs and the effect they may have both within the recipient's eye and systemically. Brunette and colleagues described the deposition of cells behind the eye lens capsule after CEC injection (Bostan et al., 2016). The capacity of CECs to cross the eye's trabecular meshwork to systemically disperse in the body appears to be unlikely (Okumura et al., 2016b). Although one patient suffered severe glaucoma after CEC injection, it was likely secondary to steroid use, and while the trabecular meshwork did not reveal blockage after gonioscopy, CECs could have been removed by macrophages that subsequently blocked drainage. Finally, it could be possible that the regeneration of the corneal endothelium in the patients with Fuchs' endothelial corneal dystrophy is due to the patient's own CECs, triggered by the ROCK inhibitor and not by

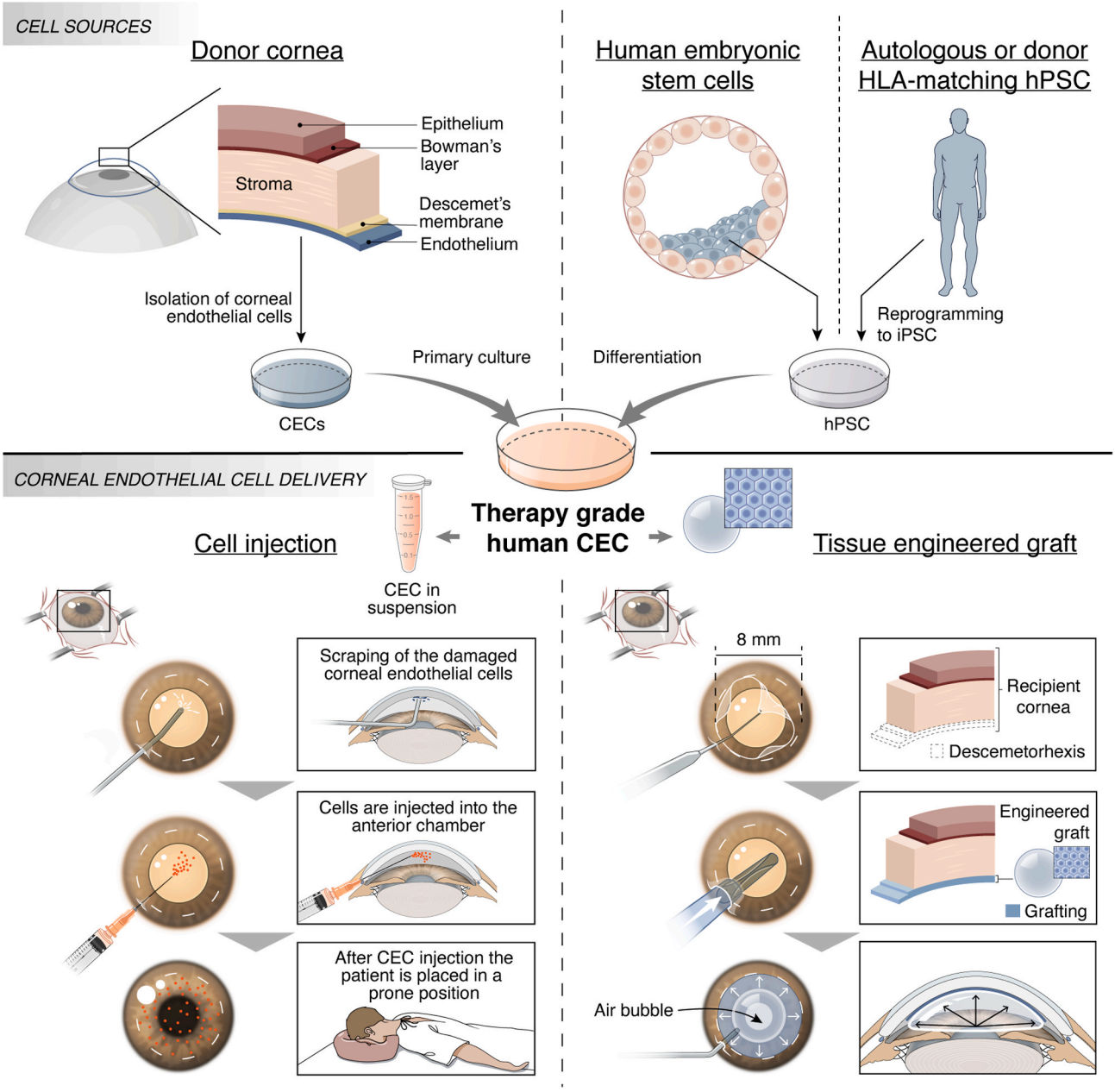


Fig. 8. Regardless of the origin of the therapy-grade CECs, whether primary cultured or pluripotent stem cell–derived, it is crucial to develop strategies to deliver them alive and with sufficient potential to adhere to the posterior part of the cornea. Currently, the approaches studied for the delivery of CECs are cell injection into the anterior chamber of the eye and the use of different substrates to bioengineer corneal endothelial grafts. This schematic overview highlights the differences of such approaches for the efficient delivery of CECs.

the injected cells. To exclude this possibility, a control group consisting of a DSO/DWEK procedure with and without ROCK inhibitor may be considered in future studies.

There are currently three ongoing clinical trials using cell injection of primary cultured human CECs worldwide, which will soon provide valuable new data to advance towards the implementation of this technology in the therapeutic setting. These comprise a phase I clinical trial (identification number NCT04191629) which studies the delivery of primary cultured CECs with ferromagnetic beads in Mexico, and two phase III clinical trials (identification numbers UMIN000034334 and UMIN000012534) to further study CEC injection in Japan.

4.2. *Tissue engineered corneal endothelium*

Another strategy to deliver CECs is the use of carriers or scaffolds to make bioengineered corneal endothelial grafts (Fig. 9). The appeal of this strategy is that the cells could be delivered to the correct place in a controlled manner, having already formed a confluent cell monolayer that is ready to start functioning. Moreover, the use of cell carriers or scaffolds would also reduce the number of cells needed compared to cell injection, thereby increasing the number of patients that could benefit from the therapy. Based on our calculations, where CECs from a single donor can be expanded to 5×10^6 to 10×10^6 cells, and considering that a corneal endothelial graft should be composed of approximately 2×10^5 CECs, we estimate that 25 to 50 patients could be treated from a single donor with this delivery method. Contrary to cell injection, where its potential to treat FECD is still uncertain, this delivery strategy could be applied to treat most corneal diseases, similar to DSAEK or DMEK. Nevertheless, this approach presents additional challenges compared to cell injection.

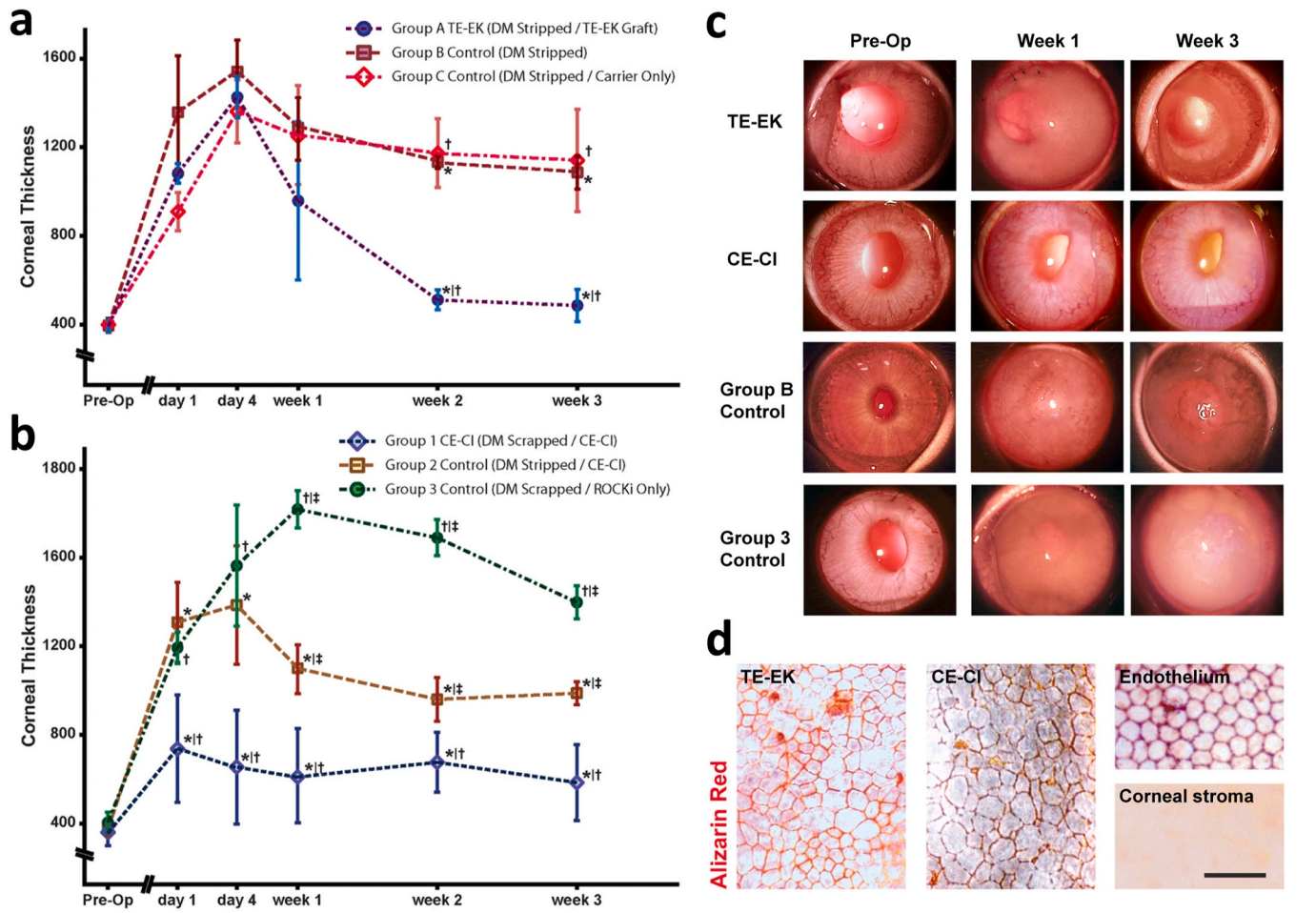


Fig. 9. The functional evaluation of two CEC delivery methods suggests that CECs can be both successfully delivered using a cell injection or a cell carrier. Primary cultured human CECs were delivered to two bullous keratopathy rabbit models using either a stromal CEC carrier (TE-EK) or a cell injection (CE-CI) and showed comparable corneal edema reduction (A and B). In Group B control the corneal endothelium was stripped without receiving any treatment. In Group C control the corneal endothelium was stripped following a transplant with the carrier without cells. In Group 2 control the corneal endothelium was stripped following an injection with CECs. In Group 3 control the endothelium was scrapped following treatment with Y-27632 ROCK inhibitor eye drops. Slit lamp images of rabbit eyes before clinical intervention (pre-op) and 1 and 3 weeks after clinical intervention show transparency recovery in corneas treated with either CE-CI or TE-EK (C). Flat-mount Alizarin red staining of rabbit corneas receiving treated with TE-EK or CE-CI show the presence of CECs mosaic (D). Sections of rabbit corneal endothelium and rabbit corneal stroma were also stained as controls (D). This Figure was adapted from Peh et al., (2019). Licensed under a Creative Commons Attribution 4.0 International License (CC BY 4.0) https://creativecommons.org/licenses/by-nc-nd/4.0/.

To bioengineer a corneal endothelial graft, the substrate or material needs to conform to specific requisites. It should be strong enough to undergo surgical manipulation without breaking, but possess a thickness comparable to DSAEK or DMEK. The selected scaffold should be transparent and its refractive index close to 1.38 to match the cornea (Patel et al., 2004), and be permeable to ions, nutrients and metabolic remnants such as lactic acid. Equally important, it needs to promote CEC adhesion and phenotype, but should also adhere to the recipients' corneal stroma. It is imperative that the selected carrier does not trigger fibrotic reactions which can damage the recipients' eye. Although not essential, biodegradability is an appealing feature so that the transplanted cells would generate their own Descemet's membrane while the carrier slowly degrades over time.

There are currently two main classifications of carriers for CECs, namely biologic scaffolds derived from tissues and synthetic or artificial scaffolds. Alternatively, some research groups are trying to develop bioengineered endothelial monolayer sheets, comprising only CECs and their extracellular matrix by culturing the cells on thermoresponsive gel substrates. In this section, we discuss the main advantages of the different strategies for bioengineering corneal endothelial grafts.

4.2.1. *Biologic scaffolds*

Biologic scaffolds are tissue-derived CEC carriers commonly generated by decellularization of biological membranes or decellularization and modification of biological matrices, resulting in a scaffold that can be used as a cell carrier (Table 1).

Bovine corneal posterior lamellae (Bayyoud et al., 2012), porcine Descemet's membrane (Diao and Hong, 2015), modified porcine corneal stroma (Zhang et al., 2014) and modified fish-scales (Parekh et al., 2018) have been used as scaffolds for CECs. Nevertheless, their xenogeneic origin might rouse skepticism because of the associated risk they could contain remnants of cellular material but also might not provide the best support for human cells.

As an alternative to xenogeneic sources, decellularized human tissues and biological membranes have also been used as scaffolds for CECs. Modified donor corneal stroma (Arnalich-Montiel et al., 2019; Choi et al., 2010; He et al., 2016a; Honda et al., 2009; Peh et al, 2017, 2019) (Fig. 10), amniotic membrane (Fan et al, 2011, 2013; Ishino et al., 2004), lens anterior capsule (Kopsachilis et al., 2012; Spinozzi et al., 2020; 2019; Van den Bogerd et al., 2018; Yoeruek et al., 2009) and Descemet's membrane (Spinozzi et al., 2020) have been used as sources for generating CEC carriers.

Table 1
Studied corneal endothelial cell carrier types, developmental stage and preparation methods.

References	Type of carrier Stage of development	Preparation method	Endothelialization
Biologic scaffolds - Decellularized tissues			
Arnalich-Montiel et al. (2019)	Human corneal stroma lamellae DSAEK in rabbits	1. Cryostat cutting (150 μm) 5–6 lamellae per cornea 2. Decellularization (SDS, DNAse)	Human. Primary culture
Bayyoud et al. (2012)	Bovine corneal stroma lamellae In vitro	1. Microkeratome cutting Undefined thickness 2. Decellularization (Tris–EDTA, SDS and Aprotinin)	Human. Primary culture
Choi et al. (2010)	Human corneal stroma lamellae In vitro	1. Microtome cutting (120–200 μm) 3-4 lamellae per cornea 2. Decellularization (Triton and NH_4OH)	Human. Primary culture
He et al. (2016)	Human corneal stroma lamellae Simulation of DSAEK on a post–mortem human eyeball	1. Femtosecond laser cutting (<100 μm) 10–12 lamellae per cornea 2. Decellularization (ethanol. SDS, DNAse I)	Human. B4G12 cell line[a]
Honda et al. (2009)	Human corneal stroma lamellae DSAEK in rabbits	1. Dissection with tissue dissection scalpel (100–150 μm) 2–3 lamellae per cornea 2. No decellularization	Human. Primary culture
Parekh et al. (2018)	Tilapia fish scale In vitro	1. Protease/surfactant/DNase/RNAse/surfactant/acetic acid/ nitric acid decalcification Average thickness: 100–120 μm	Human. Primary culture
Peh et al. (2017)	Human corneal stroma lamellae DSAEK in rabbits **Clinical Trial** (NCT04319848)	1. Femtosecond laser cutting (100 μm) Single posterior lamella with its Descemet's membrane 2. Freezing but no decellularization	Human. Primary culture
Zhang et al. (2014)	Porcine corneal stroma lamellae DSAEK in rabbits	1. Dissection with tissue dissection knives Undefined thickness 2. Decellularization (freeze-drying + air-drying)	Human. hESC-derived
Biologic scaffolds - Decellularized membranes			
Diao and Hong (2015)	Porcine Descemet's membrane In vitro	1. Microkeratome cutting + air bubble Descemet's detachment 2. Decellularization (EDTA + cell scrapping)	No endothelialization
Fan et al. (2011 **and** 2013)	Human amniotic membrane Animal experiments in cats (penetrating keratoplasty covered with endothelialized amniotic membrane)	1. Manual cutting of human amniotic membrane 2. Decellularization (trypsin–EDTA + cell scraping)	Human. Immortalized cell line
Ishino et al. (2004)	Human amniotic membrane Animal experiments in rabbits (penetrating keratoplasty covered with endothelialized amniotic membrane)	Decellularization (mechanical, EDTA)	Human. Primary culture
Kopsachilis et al. (2012)	Human crystalline lens anterior capsule In vitro	1. Manual cutting on post-mortem lens 2. Decellularization (trypsin–EDTA, distilled water)	Human. Primary culture
Spinozzi et al. (2019 **and** 2020) Telinius et al. (2020)	Human crystalline lens anterior capsule Ex vivo simulation of DMEK on human cornea on artificial anterior chamber (Spinozzi) DMEK in Gottingen minipigs (Telinius)	1. Manual cutting on post-mortem lens 2. Decellularization (ethanol, trypsin-EDTA, sponge mechanics)	Human. Primary culture
Spinozzi et al. (2020)	Human Descemet's membrane Ex vivo simulation of DMEK on human cornea on artificial anterior chamber	1. Descemet's membrane trephining (Ø 8.0 mm) and stripping 2. Decellularization (ethanol, trypsin–EDTA, sponge mechanics)	Human. Primary culture
Van den Bogerd et al. (2018b)	Human crystalline lens anterior capsule In vitro	1. Manual cutting on post-mortem lens 2. Decellularization (trypsin–EDTA, distilled water)	Human. Primary culture
Yoeruek et al. (2009)	Human crystalline lens anterior capsule In vitro	1. Manual cutting on post-mortem lens 2. Decellularization (trypsin–EDTA)	Human. Primary culture
Polymeric scaffolds – naturally occurring polymers			
Aghaei-Ghareh-Bolagh et al. (2019)	Silk fibroin and tropoelastin [m] Biocompatibility by implantation under the skin in mice	Mixture of 75% human tropoelastin and 25% silk fibroin Flat moulding and heating at 160 °C for 8 h. Thickness: 28–93 μm	Human. B4G12 line
Bourget and Proulx (2016)	Extracellular matrix self-assembled *in vitro* by keratocytes Collagens I,V,VI,XII, lumican and decorin In vitro	1. Corneal keratocyte culture of a newborn child 2. Two–layer assembly for increasing strength Thickness: 40 μm	Human. Primary culture
Choi et al. (2018)	Silk fibroin + lysophosphatidic acid[j] In vitro	Film of natural silk fibroin +8% lysophosphatidic acid Cross-linked by methanol and UV Thickness: 6–8 μm	Rabbit. Primary culture
Kim et al. (2015, 2016 **and** 2018)	- Silk fibroin + Human collagen I 2015 - Silk fibroin + aloe vera extract 2016 - Silk Fibroin + β Carotene[h]2018 In vitro (2015, 2016, 2018) DMEK in rabbits (2016)	Manufacture of a natural silk fibroin film. Coating with human Collagen I, unknown thickness 2015 Manufacture of a film composed of silk fibroin +3% aloe vera extract. Thickness: 6–8 μm 2016 Mixture of silk fibroin and β Carotene. Methanol cross-linking/ rinsing unknown thickness 2018	Rabbit. Primary culture
Kimoto et al. (2014)	Gelatin A DSAEK in monkeys	Moulding of a curved and cross-linked sheet by heating to 140 °C Thickness: 20 μm	Monkey. Primary culture
Koizumi et al. (2007)	Collagen I Vitrigel DSAEK in monkeys	Commercially available collagen I vitrigel[b] scaffold.	Monkey. Primary culture
Levis et al. (2012)	Rat tail collagen I Real Architecture For 3D Tissues (RAFT)	1. Hydrogel of 80% rat tail collagen I + 10% minimum essential medium +10% endothelial culture medium	Human. B4G12 cell line and primary culture

(*continued on next page*)

Table 1 (*continued*)

References	Type of carrier Stage of development	Preparation method	Endothelialization
	In vitro + DSAEK simulation of the material alone in a porcine eyeball *ex vivo*	2. Compression dehydration on smooth, flat plastic Thickness: 60–200 μm	
Madden et al. (2011)	Silk fibroin[d] In vitro	Manufacture of a natural silk fibroin film from the cocoons of *Bombyx mori*. Fabrication by PDMS casting. Coating with collagen IV (origin?) Thickness: 5 μm	Human. B4G12 line and primary culture
Mimura et al. (2004b)	Collagen I DSAEK in rabbits	1. Cross-linked collagen network (origin?) 2. Alkaline solution/drying/UV sterilization/rehydration Thickness: 40–50 μm	Human. Primary culture
Palchesko et al. (2016)	Collagen IV + laminin on the surface of a collagen I disc In vitro	Complex 7–step process Collagen I gel (unspecified origin), human placenta collagen IV, mouse sarcoma cell laminin. Thickness: 10 μm	Bovine and human. Primary culture
Ramachandran et al. (2020)	Silk fibroin In vitro	Manufacture of a natural silk fibroin film from the cocoons of *Bombyx mori*. Fabrication by PDMS casting. Coating with FNC coating® Thickness: 15 μm	Human. Primary and HCEnC-21T cell line
Spinozzi et al. (2019)	Collagen I Simulation of DSEK (and not DMEK because it is too sticky) on human cornea on an artificial anterior chamber	Collagen sheets (unspecified origin) Thickness: 20 μm	Porcine. Primary culture
Vazquez et al. (2016)	Human Collagen I DMEK in rabbits	Moulding of collagen I membrane extracted from clinical grade human bone. Cross-linking UV Thickness: 20 μm	Rabbit and Human. Primary Culture
Vazquez et al. (2017)	Silk Fibroin DMEK in rabbits	Manufacture of a natural silk fibroin film from the cocoons of *Bombyx mori*. Thickness: 10 μm	Rabbit and Human. Primary Culture
Watanabe et al. (2011)	Gelatin hydrogel A (porcine) In vitro	Preparation of gelatin films: drying and coating with collagen IV (origin?) Thickness: 50 μm	Human. Primary culture
Yamaguchi et al. (2016)	Atellocollagen[c] DSAEK in rabbits	Commercially available Atelocollagen hydrogel scaffold (CM-24). Coating with Viscosat®	Human. Primary culture
Yoshida et al. (2014 **and** 2017)	Atelocollagen[c] clinical-grade porcine -Biocompatibility of the material alone (without CECs) in the rabbit cornea and in the anterior chamber 2014 -DMEK in rabbits 2017	Collagen I Vitrigel by moulding a curved sheet and UV cross–linking. Atelocollagen (Nippon Meat Packers,Inc, Osaka, Japan) Thickness: 20 μm	Human. Primary culture
Polymeric scaffolds – synthetic polymers			
Chen et al. (2015)	Silk fibroin + poly (L-lactic acid-co-ε-caprolactone) (P(LLA-CL))[i] In vitro	Silk fibroin electroweaving + P(LLA–CL) (25:75) Thickness: 56 ± 4.20 μm	Human. B4G12 line
Kruse et al. (2018)	PCL and Poly(lactic-co-glycolic) acid (PLGA) † In vitro	Electrospinning of a membrane by a prototype machine PCL or PLGA solution + chloroform Disinfected with isopropanol Thickness: 109 ± 17 μm	Human. HCEC-12 line
Liang et al. (2011)	Chitosan hydrogel, hydroxypropyl chitosan (HPCTS) and sodium alginate dialdehyde. Open surgery in rabbits	Mixture of the 3 components that gels at room temperature Encapsulation of suspended endothelial cells	Rabbit. Primary culture
Ozcelik et al. (2014)	Poly (ethylene glycol) hydrogel (PEG) [k] Biocompatibility by simulation of cell–free DSEK in sheep	1. Solution of glycerol ethylate + sebacoul chloride+ α, ω-dihydroxypoly (ε–caprolactone) (PCL)+ dichloromethane 2. Cross-linked by hydrochloric acid and alcohol Thickness: 50 μm	Sheep. Primary culture
Rizwan et al. (2017)	Gelatin methacrylate (GelMa) † † In vitro Biocompatibility of the material (without endothelial cells) in the anterior chamber of rabbits	UV-crosslinked hydrogel of gelatin A and methacrylate Surface microstructured by moulding to facilitate cell adhesion Thickness: 138 ± 5 μm	Human. Primary culture
Salehi et al. (2017)	Nanofibers of poly (glycerol sebacate) (PGS) and polycaprolactone (PCL) In vitro	Electro-woven matrix manufacturing of PGS and PCL Nanofiber size: 300–500 nm Thickness unknown	Human. HCEC-12 line
Seow et al. (2019)	Agarosse corsslinked with GRGD, lysine, poly-lysine or gelatin In vitro	Mold casting of chemically cross linked agarose materials Thickness: 15–20 μm	Rabbit. Primary culture
Song et al. (2019)	Silk Fibroin + Glycerin In vitro	Film of natural silk fibroin +1% glycerol Crystallized by methanol Thickness: 7 μm	Rabbit. Primary culture
Van Hoorick et al. (2020)	Poly D-L-lactic acid (PDLLA) † † †-gelatin In vitro	Multi-step spin coating of: Gelatin A, PDLLA, gelatin B. Membrane harvesting by gelatin A solution in 40 °C water bath Thickness: 0.8–1 μm	Human. B4G12 line
Wang et al. (2012)	Chitosan[e] + polycaprolactone (PCL)[f] In vitro	1. Mixture of 75% chitosan and 25% polycaprolactone 2. NaOH drying/neutralization/rinse/ethanol 70%/UV sterilization	Bovine. Primary culture
Young et al. (2014)	Chitosan + polycaprolactone (PCL) In vitro	Preparation of chitosan + polycaprolactone solutions/ evaporation/NaOH treatment/70% ethanol sterilization/UV treatment/Rinsing	Bovine. Primary culture

(*continued on next page*)

Table 1 (*continued*)

References	Type of carrier Stage of development	Preparation method	Endothelialization
Bioengineered endothelial monolayer sheets			
Hsiue et al. (2006) **Lai et al. (2013)**	Gelatin A[g] Animal experimentation in rabbits	Cell culture on pNIPAAm thermoresponsive gel to generate ultrathin corneal endothelial grafts Gelatin disc used as transplant substrate (Thickness: 700–800 μm) Endothelial sheet glued upside down on the disc and inserted to have the cells directly against the cornea. The gelatin is then resorbed	Human. Primary culture
Ide et al. (2006)	NONE In vitro	Cell culture on poly(N–isopropylacrylamide) (pNIPAAm), which is heat-sensitive and allows the endothelium to be detached in an ultrathin corneal endothelial graft by lowering the temperature to 20 °C	Human. Primary culture
Lai et al. (2015)	Recombinant hyaluronic acid Animal experimentation in rabbits	Cell culture on PIPAAm thermoresponsive polymer to generate ultrathin corneal endothelial grafts Cross-linked hyaluronic acid disc used as transplant substrate (Thickness: 700 μm) Endothelial sheet glued upside down on the disc and inserted to have the cells directly against the cornea. The hyaluronic acid is then resorbed	Rabbit. Primary culture
Madathil et al. (2014)	NONE In vitro	Cell culture on NGMA thermoresponsive polymer (pNIPAAm + glycydyl methacrylate), which is heat-sensitive and allows the endothelium to be detached in an ultrathin corneal endothelial graft by lowering the temperature to 20 °C	Rabbit. Primary culture
Sumide et al. (2006)	NONE In vivo. PK in rabbit models, ultrathin endothelial graft is attached on recipient's dissected corneal stromal bed and then re-inserted in the recipient eye	Cell culture on poly(N–isopropylacrylamide) (pNIPAAm), which is heat-sensitive and allows the endothelium to be detached in an ultrathin corneal endothelial graft by lowering the temperature to 20 °C	Human. Primary culture
Teichmann et al. (2013 and 2015)	NONE Lamina coating and chondroitin sulfate In vitro	Cell culture on a heat-sensitive support of poly (vinyl methyl ether) (PVME) and vinyl methyl ether and maleic acid (PVMEMA) coated with laminin, chondroitin–6–sulfate and cyclo (arginine–glycine–aspartic acid–D–tyrosine-lysine) cRGD peptides. Ultrathin corneal endothelial graft is detached by lowering the temperature to 20 °C	Human. HCEC-12 line

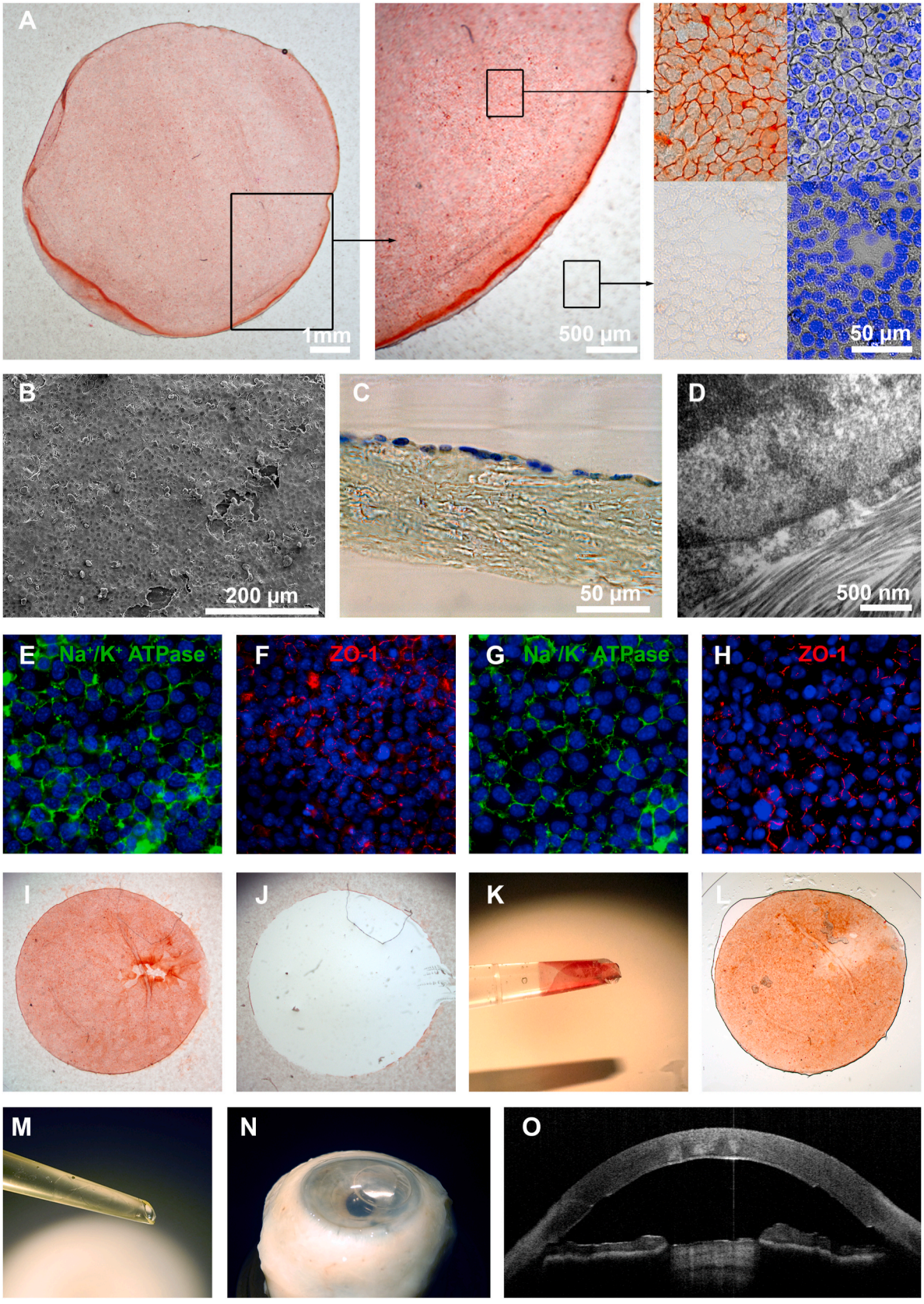
[l]hyaluronic acid: glycosaminoglycan component of the extracellular matrix that contributes to cell proliferation and migration.
[a] B4G12 and HCEC-12 are the two human corneal endothelial cell lines commercially available. https://www.dsmz.de.
[b] Vitrigel: gelled and vitrified collagen solution by dehydration at controlled temperature to allow the formation of very high density collagen fibrils. The result is the formation of a translucent or transparent resistant material which can be later rehydrated.
[c] Atelocollagen: type I collagen depleted by enzymatic treatment of its 2 extremities (telopeptides) resulting in a product with reduced immunogenicity.
[d] Silk fibroin: insoluble proteins from silk of insects, including arachnids and silkworms.
[e] Chitosan: biodegradable linear polysaccharide chemically extracted from chitin, which forms the exoskeleton of crustaceans, arthropods and the walls of certain fungi.
[f] Polycaprolactone (PCL): biodegradable polyester used in particular in certain sutures or sustained-release medicines.
[g] Gelatin: mixture of proteins obtained by partial hydrolysis of collagen. Type A is extracted from porcine skin and bones.
[h] β Carotene: vegetal pigment precursor of vitamin A.
[i] Poly (L–lactic acid–co–ε–caprolactone): biodegradable copolymer obtained from L–lactic acid and ε-caprolactone.
[j] Lysophosphatidic acid: cellular endogenous mitogenic glycerophospholipid.
[k] poly (ethylene glycol) (PEG): water-soluble and biodegradable polyether compound.
[m] Tropoelastin: 60–70 kDa monomer, precursor of elastin, key protein of the mammalian extracellular matrix.
[†] Poly(lactic-co-glycolic) acid (PLGA): biodegradable copolymer obtained from L–lactic acid and glycolic acid.
[††] Gelatin methacrylate (GelMa): photoporymelizable matrix synthetically derived from hydrolitical degradation of collagen.
[†††] Poly D-L-lactic acid (PDLLA): biodegradable polymer generated from a racemic mixture of L–lactic acid and D-lactic acid.

Considering data from pre-clinical studies, stromal scaffolds have been the most successful in reverting corneal edema and recovering cornea transparency in rabbits (Honda et al., 2009; Peh et al, 2017, 2019). Conversely, lens anterior capsule carriers triggered strong fibrotic reactions in minipig eyes' (Telinius et al., 2020) and amniotic membranes did not fully revert corneal edema in cats (Fan et al., 2013). There is currently an ongoing phase I clinical trial in Singapore (identification number NCT04319848) for delivering primary cultured CECs using decellularized and modified human corneal stromal carriers to patients suffering from corneal endothelial disease. This study is at an early recruitment phase and more information will be available in the coming years.

The attraction of using biological scaffolds rests with their somatic origin, as there is no better biocompatibility than that of a native tissue. Nevertheless, biological carriers present some other hurdles. Generating CEC carriers from donor tissue will always be dependent on donor availability and require adequate tissue banking. Countries lacking this infrastructure may have difficulties in using tissue-derived CEC carriers. Furthermore, the donor-to-donor variability may influence the final characteristics of the generated carriers and it is uncertain of how this will affect the final therapy.

4.2.2. Polymer scaffolds

Polymer scaffolds are CEC carriers that can be generated from naturally occurring polymers derived from biological sources or from synthetic polymers. Using different approaches such as crosslinking (Maitra and Shukla, 2014), spin-coating (Lawrence and Zhou, 1991), and electrospinning (Schiffman and Schauer, 2008), these materials can be fabricated into CEC carriers with properties resulting from both the polymer and the fabrication techniques (Table 1). The main advantages of polymer scaffolds are the independence from donor tissue and the ability to generate an abundant amount of material in a rapid and reproducible way to create a highly defined product for clinical use. Nevertheless, using carriers from non-physiological origins may result in



(caption on next page)

Fig. 10. Bioengineered human corneal endothelial graft. Human CEC stained with Alizarin Red on a human stromal cell carrier (A). Only the CECs grown on the stromal carrier show the characteristic red staining caused by calcium deposits in the tight junction regions of the bioengineered endothelial graft. Cell nuclei were stained with Hoechst 33342. Scanning electron microscopy (B) and semi-thin sections stained with toluidine blue (C) showed a uniform cell monolayer on the stromal cell carrier. Transmission electron microscopy confirmed the adherence of the CEC on the stromal collagen fibers (D). Na^+/K^+ ATPase and ZO-1 immunofluorescence staining were comparable in CECs grown in tissue culture plastic (E and F) and CECs grown on the human stromal carrier (G and H). (I) Shows another bioengineered endothelial graft stained with Alizarin Red. The bioengineered endothelial graft could be successfully detached from the culture tissue plastic (J), rolled and loaded in an insertion cartridge (K), and released through the narrow opening of the insertion cartridge (L) without showing morphological alterations. After culture of CEC the bioengineered graft was loaded in an injector (M) and grafting was simulated in an *ex vivo* cadaver human eye globe (N). OCT measurement revealed that the bioengineered graft followed and adjusted to the posterior curvature of the recipient cornea (O). This figure was obtained from He et al., (2016a). Licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (CC BY-NC-ND 4.0) https://creativecommons.org/licenses/by-nc-nd/4.0/.

adverse reactions.

Commonly used naturally occurring polymers have been collagen, including collagen vitrigel, (Bourget and Proulx, 2016; Koizumi et al., 2007; Levis et al., 2012; Mimura et al., 2004b; Palchesko et al., 2016; Spinozzi et al., 2019; Vazquez et al., 2016; Yamaguchi et al., 2016; Yoshida et al, 2014, 2017), collagen-derived gelatin (Kimoto et al., 2014; Watanabe et al., 2011), and silk fibroin (Aghaei-Ghareh-Bolagh et al., 2019; Choi et al., 2018; Kim et al., 2018; 2016; 2015; Madden et al., 2011; Ramachandran et al., 2020; Vazquez et al., 2017). The data have been variable, and animal experiments have revealed the main shortcomings of these carriers. For example, collagen carriers have difficulties staying attached to the recipients' corneal stroma, and the scaffolds tend to detach after 2 weeks (Koizumi et al., 2007). Other studies with collagen-derived carriers did not monitor carrier integration beyond two weeks after implantation (Yoshida et al, 2014, 2017) leaving the graft integration question unanswered.

Similarly, gelatin-based scaffolds also present integration problems; a study reported that four weeks after implantation, 60% of the transplanted scaffolds detached from the recipients' corneal stroma (Kimoto et al., 2014). Silk fibroin scaffolds have also been tested in animal models, nevertheless they triggered fibrotic reactions on recipient rabbits' corneal stroma (Vazquez et al., 2017) making them ineligible for therapeutic use. There is an urgent need to overcome the previously mentioned shortcomings of these carriers in order to translate them into the clinical setting.

Many synthetic polymers have also been studied as CECs carriers. Examples of synthetic polymers used have been polycaprolactone (PCL) (Kruse et al., 2018), poly(ethylene glycol) (PEG) (Ozcelik et al., 2014), polylactic acid (PLA) (Van Hoorick et al., 2020), chitosan (Liang et al., 2011), gelatin methacrylate (GelMA) (Rizwan et al., 2017), chemically modified agarose (Seow et al., 2019), and combinations of polymers such as silk fibroin–glycerin (Song et al., 2019) and chitosan–PCL (Wang et al., 2012; Young et al., 2014). These synthetic approaches purport to allow a degree of design flexibility of the material to fit the clinical purpose as well as offering the consistency that biologic scaffolds cannot.

Despite promising results in terms of the physical properties of the carrier, cell survival, adherence, and phenotype maintenance, there is only one *in vitro* study performed using primary cultured CECs (Rizwan et al., 2017). The biocompatibility characterization of the other studied carriers has been performed by using immortalized cell lines (J. Chen et al., 2015; Kruse et al., 2018; Salehi et al., 2017; Van Hoorick et al., 2020) or primary cultured CECs from non-human origin (Liang et al., 2011; Song et al., 2019; Wang et al., 2012; Young et al., 2014). To better understand if the generated carriers are a good platform for regenerative medicine, it is compulsory to demonstrate biocompatibility and phenotype maintenance with primary cultured human CECs. Animal experiments are crucial to demonstrate that the generated scaffolds can integrate in the recipient's corneal stroma without triggering a fibrotic reaction.

4.2.3. Bioengineered endothelial monolayer sheets

Conversely, different groups are studying the possibility to deliver CECs in the form of bioengineered endothelial monolayer sheets. The appeal of this method is that the grafts are assembled by the cells' own extracellular matrix, generating a fully biocompatible construct with a decreased risk of adverse reactions upon implantation (Table 1).

To generate such sheets, CECs are cultured on thermoresponsive polymer substrates. After cell confluency has been reached, and through a decrease of temperature, the CEC monolayer is detached from the thermoresponsive culture surface, resulting in a highly compacted cell sheet, or ultrathin corneal endothelial graft (Ide et al., 2006; Lai et al., 2006; Madathil et al., 2014; Sumide et al., 2006; Teichmann et al, 2013, 2015). These bioengineered endothelial monolayer sheets appear to be an elegant approach to deliver CECs into the cornea. Moreover, they present a biocompatibility advantage compared to the cell carriers. However, these ultrathin cell sheets are particularly fragile and their manipulation inside the eye can be technically challenging. It is of utmost importance to develop techniques to enable their accurate and reproducible delivery into the recipient cornea. Loading such sheets on gelatin or hyaluronic acid carriers has been proposed to reduce their manipulation (Hsiue et al., 2006; Lai et al, 2013, 2015). Nevertheless, this approach shares similarities with the use of CEC carriers and their potential biocompatibility and integration problems.

5. Acellular corneal endothelial graft substitutes

Development of surgical techniques such as DSO or DWEK revealed that in specific cases of FECD, the regeneration of corneal endothelium could be achieved without the use of a donor endothelial graft, challenging the current state-of-the-art. Nevertheless, DSO/DWEK are limited to early-stage disease in relatively young patients and recovery is long and unpredictable. Endothelial graft substitutes comprising of synthetic or tissue-derived matrices could aim to promote corneal healing when implanted after DSO/DWEK procedures. With this technique, no donor graft would be required. The potential advantage is that acellular corneal endothelial graft substitutes may promote or facilitate proliferation and migration of peripheral CECs to repopulate acellular regions, and also support corneal deturgescence and edema reduction upon implantation.

Mehta and colleagues were the first to report the use of acellular corneal endothelial graft substitutes in 2017 (Bhogal et al., 2017). In their study, the corneal endothelium was stripped off rabbit eyes and decellularized human Descemet's membrane was introduced similarly to a DMEK procedure, a process called Descemet membrane transfer. The animal group receiving allogenic decellularized Descemet's membrane showed an increased corneal endothelial migration and a faster edema reduction compared to the control group which did not received an allogeneic transplant (Fig. 11). There is currently an ongoing clinical trial in Singapore studying such technique in humans (identification number NCT03275896). The first clinical results were recently published, and the first transplantation of a 4 mm diameter decellularized Descemet's membrane into a patient was successful in improving the patient's best-corrected Snellen visial acquity from 6/18 to 6/7.5 at 6 months after transplant (Soh and Mehta, 2018). Moreover, corneal thickness was reduced from 603 μm to 569 μm and central CEC density was 889 cells/mm^2 (Soh and Mehta, 2018). This first proof-of-concept study paves the way to study acellular corneal endothelial graft substitutes to promote CEC healing and edema reduction.

A second approach proposed the use of a synthetic graft substitute to

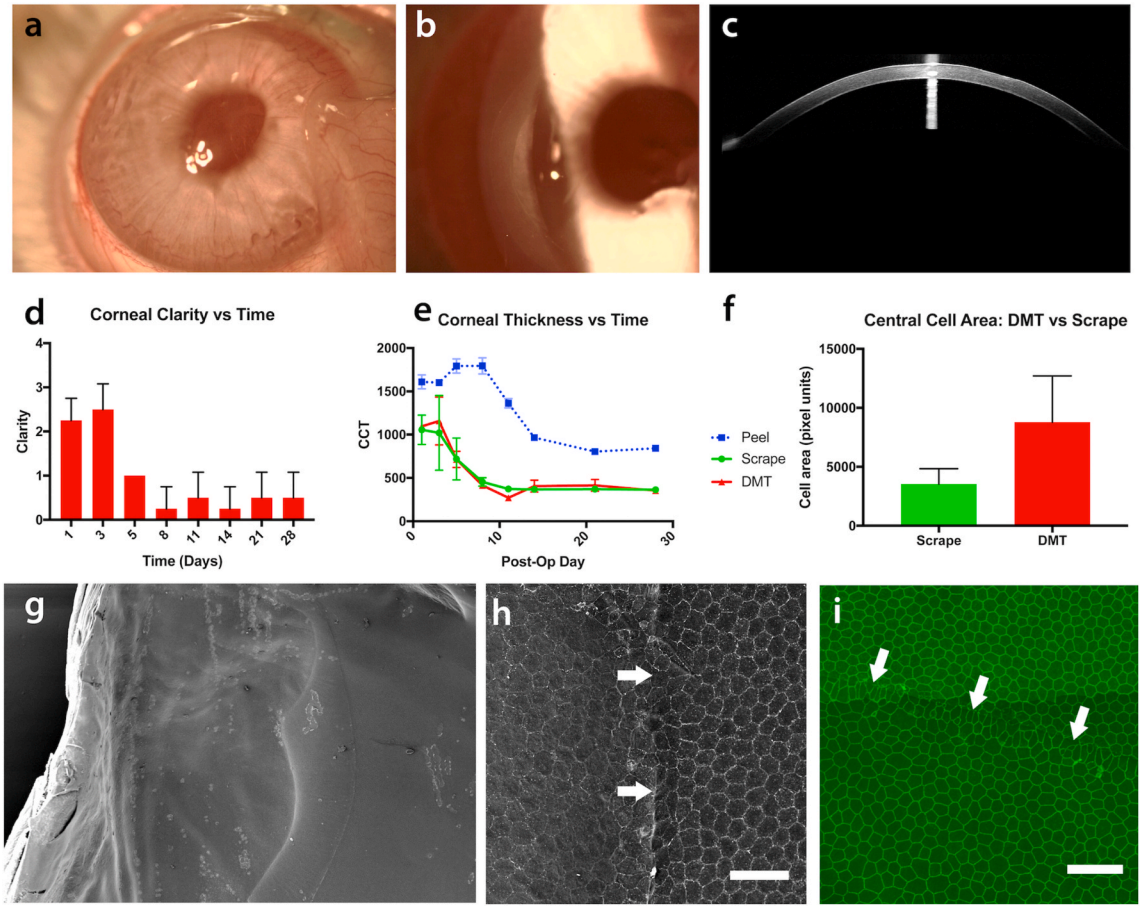


Fig. 11. Descemet's membrane transfer (DMT) on a rabbit model. Eleven days after DMT the corneas appeared to be clear in the center (A). A small opaque area could be seen in the periphery of the Descemet's graft, at the descemetorhexis edge (B). Optical coherence tomography showed that corneal thickness of rabbits receiving a Descemet's membranes transplant did not differ from control animals (C). Rabbit eyes showed a central corneal clarity of 0 or 1 8 days after DMT (D). Corneal thickness recovery of eyes receiving DMT was comparable to the animal control group were the cells were scraped, leaving a region of the Descemet's membrane denuded (scrape) and significantly better compared to the animal control group that underwent a descemetorhexis (peel) (E). Central endothelial cell area was greater in the corneas receiving DMT compared to the scrape group (F). Scanning electron microscopy revealed that CECs could migrate over the Descemet's membrane graft, forming a complete monolayer (G). CEC bridged over the edge of the Descemet's membrane graft edge, indicated with white arrows (H). Immunofluorescence analysis of ZO-1 revealed that the bridging CECs formed an uninterrupted cell monolayer over the transferred Descemet's membrane (I). Scale bar: 50 μm (H) and 75 μm (I). This figure was obtained from Bhogal et al., (2017). Licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (CC BY-NC-ND 4.0) https://creativecommons.org/licenses/by-nc-nd/4.0/.

reverse corneal endothelial disease. There is currently an ongoing randomized multi-center clinical trial (identification number NCT03069521) aimed to evaluate if a Contomac Ci26-based implant (EndoArt), is successful in reversing corneal edema and promoting sight recovery. The EndoArt implant was designed to prevent fluid infiltration into the cornea, thereby preventing and reversing corneal edema. While the data on this study are still restricted, preliminary results from eight patients with chronic corneal edema are encouraging (Daphna and Marcovich, 2020). Out of these eight patients, seven presented a reduction of corneal edema and recovery of transparency after EndoArt implantation. In one patient, surgery failed due to hypotomy and a rescue penetrating keratoplasty was performed. In the seven successful surgeries the construct detached from the recipients' cornea and had to be repositioned by rebubbling until correctly attached to the cornea (Fig. 12). The cornea remained clear in the seven patients for up to 4 months.

Despite initial positive results, the exact timespan over which the cornea will remain transparent and whether corneal edema will reoccur remains uncertain. Furthermore, more data are needed to determine the precise detachment rate and if corneal nutrition will be affected in the long-term due to the impermeability of the construct. Improvements in the material attachment to the recipients' cornea would be required to

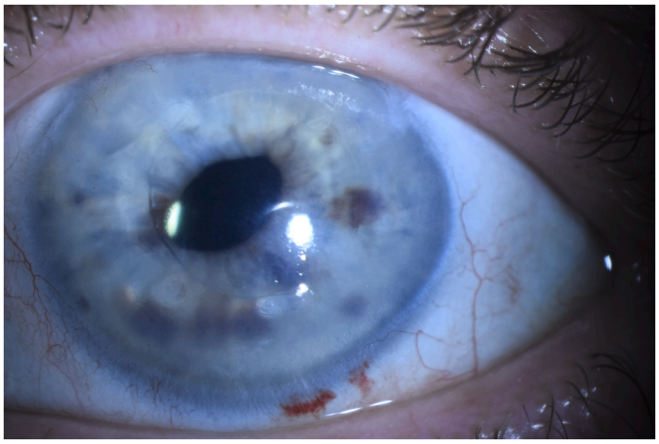


Fig. 12. Slit lamp image of a patient's eye one day after EndoArt implantation. Courtesy of Dr Ruth Lapid-Gortzak, Amsterdam University Medical Center, the Netherlands.

enable widely acceptance of such device. Moreover, based on currently available data, CECs are unable to migrate over the EndoArt, the effect of which remains to be seen. Overall, although acellular graft substitutes offer an attractive solution to current tissue shortage, future studies are needed to determine if they are successful enough to be implemented in a large scale clinical setting and for what indication.

6. Pharmacological modulation of the corneal endothelium

The pharmacological modulation of the corneal endothelium to promote cell survival, proliferation and migration has also been studied as a potential treatment for corneal endothelial disease, showing promising preliminary results (Table 2). The appeal of this therapeutic modality is that the patients' endothelium could regenerate with a minimal procedure of intracameral or topical delivery of a drug. While it might seem that the risks of using a pharmacological modulation of the corneal endothelium are rather low, as it would always be possible to perform a rescue DMEK/DSAEK if the patients are not recovering after a reasonable treatment time window to avoid subepithelial or stromal scarring, it is important to monitor possible infiltrations of the trabecular meshwork or development of iridocorneal endothelial type syndrome caused by the therapeutic drug.

ROCK inhibitors are one of the most promising candidates for treating corneal endothelial disease. ROCK is a protein kinase downstream of the effector GTPase Rho, which plays a crucial role in cytoskeleton regulation. The first drug candidate identified was Y-27632, which showed potential to trigger CEC repair and survival *in vitro* (Okumura et al., 2009; Pipparelli et al., 2013). After showing success in reducing corneal edema and recovering visual acuity in rabbit and monkey bullous keratopathy models (Koizumi et al., 2013; Okumura et al., 2013b), a first clinical trial was performed in Japan (identification number UMIN000003625). This trial comprised two different groups. First, a group of eight patients were treated, four with FECD and four with bullous keratopathy. Briefly, the damaged CECs were surgically removed gently, preserving the Descemet's membrane. After this minimal surgical procedure, the eight patients were treated with topical delivery of 10 mM Y-27632 using eye drops six times a day for 7 days. Cornea thickness was reported at 3 and 6 months after treatment. In the four patients suffering from central corneal edema caused by FECD, a decrease of corneal thickness, from an average of 740 μM to an average of 640 μm was reported after 6 months. Conversely, the corneal thickness did not reduce after the treatment with Y-27632 in the four patients with bullous keratopathy and diffuse corneal edema (Koizumi et al., 2013; Okumura et al., 2013b). The second group consisted of three patients suffering from bullous keratopathy after cataract surgery, where the Descemet's membrane was partially detached and lost. These patients were directly treated with topical delivery of 1 mM Y-27632 six times a day for 4 months and four times a day for 2 months using eye drops (Okumura et al., 2015). After 3 months of treatment, the corneal edema was reduced from values of 900–610 μm to values of 580–503 μm and visual acuity recovered to 20/20 in 2 patients and 20/25 in one patient (Okumura et al., 2015). These studies implied the success of the therapy depends on the disease background and highlight the importance of developing a controlled dosage and treatment duration.

Ripasudil, another ROCK inhibitor, has also shown success in reducing corneal edema and recovering corneal clarity in a bullous keratopathy rabbit model (Okumura et al., 2016a). Y-27632 and Ripasudil target the ATP-dependent kinase domains of ROCK1 and ROCK2 with an IC_{50} of 0.11 M and 0.051 M for ROCK1 and 0.17 M and 0.019 M for ROCK2 respectively (Isobe et al., 2014). The increased efficacy and affinity of Ripasudil compared to Y-27632 for ROCK1 and ROCK2, indicated by the lower IC_{50}, is due to the addition of a fluorine atom in the isoquinoline moiety (Kaneko et al., 2016; Liao et al., 2007). Both ROCK inhibitors show a comparable ocular distribution reaching the highest concentration in the cornea 15–30 min after instillation (Isobe et al., 2014; Challa et al., 2009). *In vitro* studies suggest that ripasudil might promote an increase in cell proliferation, migration and adhesion (Schlötzer-Schrehardt et al., 2020), thus causing the regeneration of the corneal endothelium. In 2016, a first-in-human study was performed in Australia. Two patients with FECD who underwent a DSO/DWEK procedure and did not experience corneal clearing after 2 and 3 months were treated with topical delivery of ripasudil 0.4% eye drops 6 times a day for 2 weeks. One month after treatment, the CECs had repopulated the bare stroma of both patients and corneal opacity was reduced (Moloney et al., 2017). In the same study, another eye that did not clear 2 months after a DSO/DWEK procedure was treated with the topical delivery of 10 μM Y-27632 using eye drops six times a day for 2 weeks without success.

ROCK inhibitors have shown success in clinical trials, but the number of patients involved in these studies is very low, though there are a number of studies currently recruiting. In order to conclude if ROCK inhibitors have a beneficial effect treating corneal endothelial disease,

Table 2
Studied drugs and their developmental stage for the treatment of corneal endothelial disease.

Drugs for the treatment of corneal endothelial disease					
Drug	**Drug class**	**Possible mechanism of action**	**Developmental stage**	**Ongoing clinical trials**	**References**
Y-27632	Inhibitor of Rho-associated, coiled–coil–containing, protein kinase 1	Increase in CEC survival, proliferation and/or migration	In humans Completed clinical trial: UMIN000003625	No	Okumura et al., (2013b), 2015 (Koizumi et al., 2013)
Ripasudil	Inhibitor of Rho–associated, coiled–coil–containing protein kinase 1	Increase in CEC survival, proliferation and/or migration	In humans	Yes NCT03575130, NCT03813056, NCT04250207, and NCT03249337	Okumura et al. 2016 Moloney et al. (2017) Schlötzer-Schrehardt et al. (2020)
TTHX1114	Engineered human fibroblast growth factor 1 protein	Promotion of CEC proliferation	In humans	Yes NCT04520321	Xia et al. (2012)
SB431542	Inhibitor of the TGF–β type I receptors ALK5, ALK4 and ALK7	Decrease of endothelial to mesenchymal transition	In vitro (immortalized human CEC lines)	No	Okumura et al. (2017)
Sulforaphane	Nrf2 transcription factor activator	Oxidative stress and apoptosis reduction	In vitro (immortalized human CEC lines)	No	Ziaei et al. (2013)
N-acetyl cysteine (NAC)	Scavanger of reactive oxygen species	Oxidative stress and apoptosis reduction	In vitro (immortalized human CECs) In vivo (early onset FECD mouse model)	No	Halilovic et al. (2016) Kim et al. (2014) Liu et al. (2020)
Oxotremorine	Selective muscarinic acetylcholine receptor agonist	Oxidative stress and apoptosis reduction	In vitro (bovine CECs)	No	Kim et al. (2017)
Mefenamic acid	Nonsteroidal anti-inflammatory drug inhibitor of cyclooxygenase 1 and 2	Oxidative stress and apoptosis reduction	In vitro (bovine CECs)	No	Kim et al. (2017)

there is the urgent need to perform larger randomized control trials with a DWEK control group and specifically defined dosing and therapeutic length.

Furthermore, there is the need to understand each disease case such that we are able to identify which patients will benefit from the therapy and which patients are not suitable candidates. Crucial considerations could be patient age, genetic background, the stage of the disease, the absence/presence or amount of bullae, the peripheral CEC density, and the shape and size of the Descemetorhexis. Part of this challenge is that the biological action of ROCK inhibitors is not well understood, for example whether they increase cell survival, proliferation or migration.

There are currently four ongoing phase II, randomized and double-blinded clinical trials using topical delivery of Ripasudil for treating FECD. A first study in Germany (identification number NCT03575130) will involve 21 participants and study the topical delivery of ripasudil 0.4% eye drops after a DWEK procedure six times per day for 2–4 weeks. The control group will undergo a DWEK procedure and will be treated with placebo artificial tears. A second clinical trial in the United States (identification number NCT03813056) will involve 72 participants and will study the benefits of Ripasudil 0.4% eye drops delivered six times per day for 2–4 weeks after a DMEK procedure. The control group will undergo a DMEK procedure and will be treated with placebo artificial tears. A third study (identification number NCT03249337) will compare Ripasudil dosing regimen of 3 times a day with 6 times a day in patients who underwent a DSO/DWEK procedure for FECD. Finally, a fourth international multicenter trial (identification number NCT04250207) will involve 60 participants and will study the topical delivery of two different doses of Ripasudil (K-321 solution) eye drops after a DWEK procedure on FECD patients. Half the controls that will undergo DWEK will receive placebo only and the other half will receive twice daily placebo and twice daily Ripasudil. These studies will give more insight into the use of ROCK inhibitors for treating corneal endothelial disease.

To date, ROCK inhibitors have been the most studied drugs for treating corneal endothelial disease, but there is also promising research exploring other pharmacological tools to promote corneal endothelial regeneration. Different growth factors such as epidermal growth factor (Hoppenreijs et al., 1992), platelet-derived growth factor (Hoppenreijs et al., 1994) and fibroblast growth factors (Lu et al., 2006) have been studied to promote migration and proliferation of CECs for tissue regeneration. Nevertheless, their potential benefit comes with the risk of causing an undesired EndMT (Roy et al., 2015; Wendt et al., 2012). Research in this field identified an engineered FGF-1 molecule, TTHX1114 (Xia et al., 2012), which has shown potential *in vitro* and *in vivo* to trigger corneal endothelial regeneration without any relevant side effects (United States patent registry number US, 2016/0263.190 A1). There is currently an ongoing phase I/II clinical trial in the United States (identification number NCT04520321) studying the safety and efficacy of TTHX1114 for treating corneal endothelial disease. This study is at an early recruitment phase.

There has also been research specifically focusing on the pharmacological modulation of FECD. It is known that the extracellular environment of FECD increases the risk of an endothelial to mesenchymal transition leading to a loss of function of the corneal endothelium (Kocaba et al., 2018). One of the better understood factors causing this transition is the increase of TGF-β (Roy et al., 2015; Wendt et al., 2012). TGF- β inhibitors can reduce the endothelial to mesenchymal transition of CECs *in vitro* (Okumura et al., 2017) suggesting the potential of these pharmacological tools to treat patients with FECD.

Another characteristic of the pathological profile of FECD is the cell death due to an increase in oxidative stress (Jurkunas et al., 2010). N-acetyl cysteine (NAC), a scavenger of reactive oxygen species, has been shown to reduce CEC death *in vitro* and in a transgenic *COL8A2*$^{L450W/L450W}$ mouse model (Halilovic et al., 2016; Kim et al., 2014) and CEC ultraviolet damage model (Liu et al., 2020). Nevertheless, the *COL8A2* transgenic model does not develop corneal edema and the animal model developed by Liu et al. is a UV damage model, not a model of FECD. Sulforaphane has also been identified as an oxidative stress reducer by phosphorylating and activating Nrf2, a transcription factor that promotes expression of antioxidative stress proteins, and has been shown to decrease CEC apoptosis *in vitro* (Lovatt et al., 2020; Ziaei et al., 2013). Oxotremorine, a selective muscarinic acetylcholine receptor agonist, and mefenamic acid, a nonsteroidal anti-inflammatory drugs, have also shown to decrease oxidative stress and increase survival in human CECs *in vitro* (Kim et al., 2017). These data suggest that pharmacological tools could be used as a potential treatment for Fuchs' endothelial corneal dystrophy. However, there is a clear need for double blinded randomized controlled clinical trials to generate higher level evidence.

7. Genetic modulation of the corneal endothelium

One of the leading causes of corneal endothelial disease are genomic alterations in patients, which the gene or protein subsequently affect CECs. Developing tools for correcting these genetic alterations or avoiding their associated effects could potentially reduce the need for corneal transplantation, making more corneal donor tissue available for other purposes.

There are currently four corneal endothelial dystrophies with a clear genetic origin, namely: polymorphous corneal dystrophy (PPCD), congenital hereditary endothelial dystrophy (CHED), X-linked endothelial dystrophy (XCED), and FECD (Aldave et al., 2013). The first three are rare (Aldave et al., 2013), while FECD, a disease of autosomal dominant nature with incomplete penetrance, has a global estimated prevalence of 4–5% in people above 40 years old (Fautsch et al., 2020) and is the leading indication for corneal transplantation worldwide (Ong et al., 2020). The development of a genetic modulation therapy to specifically treat FECD could have a major impact on reducing the need for corneal donor tissue.

The pathophysiology of FECD has been extensively reviewed elsewhere (Fautsch et al., 2020; Ong et al., 2020). Although alterations in different genes, among them *SLC4A11*, *ZEB1* or *COL8A2*, have been associated with the disease, the most common genetic alteration is an intronic CTG trinucleotide repeat expansion in the transcription factor 4 (*TCF4*) gene (Fautsch et al., 2020; Ong et al., 2020). The role of the CTG repeat expansion has been thoroughly reviewed elsewhere (Fautsch et al., 2020). The CTG repeat expansion has a prevalence in Fuchs' patients ranging from 26% to 79%, depending on group ethnicity (Fautsch et al., 2020), which positions the CTG trinucleotide repeat expansion in the *TCF4* gene as the most viable genetic target for developing a genetic modulation therapy. While there is not yet a clearly identified genetic mechanism to explain the effect of this trinucleotide expansion on the *TCF4* gene, there is evidence for three hypotheses: a dysregulated TCF4 protein expression leading to a protein loss–of–function, RNA repeat–mediated toxicity, or toxic repeat peptide generated by repeat–associated non–AUG dependent (RAN) translation (Fautsch et al., 2020). In this section, we discuss the possible therapeutic approaches for corneal endothelial genetic modulation based on the altered *TCF4* gene origin of FECD.

7.1. Gene augmentation

Gene augmentation consists of the delivery of a functioning copy of a specific defective gene aimed to correct a disease caused by a protein loss of function. The most commonly used systems for nucleic acid transfer have traditionally been viral vectors, such as adeno associated viral vectors (AAVs) and adenoviral vectors (AVs) (Robbins and Ghivizzani, 1998). AAV serotypes AAV-7, AAV-8 and AAV-9 have shown strong tropism for ocular tissues (Lebherz et al., 2008), being good candidates for such approach. The size of *TCF4* messenger RNA, around 8000 bp, is a key consideration for developing viral delivery methods. *TCF4* can be too large to be delivered with specific AAV serotypes (Wu et al., 2010). Specific AAV serotypes, namely AAV-5 ave been successful

in delivering genes up to 8900 bp (Allocca et al., 2008) and could be used to deliver a functioning copy of *TCF4* messenger RNA to CECs. Non-viral delivery strategies such as liposomal gene delivery or DNA–-protein conjugates have also been studied (Robbins and Ghivizzani, 1998). Lessons learnt from gene augmentation therapies focused on treating other eye diseases (Moore et al., 2018) could facilitate the generation of a gene augmentation therapy if the corneal endothelial disease origin was closely related to an altered protein expression. Given that the cells are arrested in G1 phase, the CECs are an attractive target for gene therapies as the cells do not divide and are therefore more likely to retain their delivered material. Furthermore, the immune-privileged nature of the eye provides an advantage of likely allowing the repeated delivery of gene therapy products. Gene augmentation studies on retinal congenital blindness due to *RPE65* deficiency have shown that the repeated subretinal administration of an AAV-based gene therapy in the contralateral eye did not cause immune reactions even if the recipient presented circulating anti-AAV antibodies (Annear et al., 2011; Bennett et al., 2012). Other studies have also assessed the low presence of AAV neutralizing antibodies in the aqueous humor in humans (Andrzejewski et al., 2021; Lee et al., 2019) and after the subretinal delivery of AAV-based gene therapy treatment in dogs (Amado et al., 2010). These data suggest that repeated delivery of a gene therapy product in the anterior chamber is unlikely to generate immune reactions that could affect the therapy efficacy or the recipient's eye. Nevertheless, further studies are required to understand the possible immune reactions following repeated administration of gene therapy products in the anterior eye chamber.

7.2. Antisense oligonucleotide-based modulation

Antisense oligonucleotides are a strategy for treating genetic diseases caused by either RNA repeat–mediated toxicity or the generation of toxic repeat peptides by RAN translation. Antisense oligonucleotides such as small interference RNA (siRNA) or micro RNA (miRNA) are complementary sequences to messenger RNA (mRNA) that trigger their blockage or elimination (Rinaldi and Wood, 2018). Designing specific antisense oligonucleotide strategies targeting the mRNA transcripts containing the CUG trinucleotide expansion would allow the removal of their associated deleterious effects, as only the non-expansion containing allele would be translated.

Three reports have studied the use of antisense oligonucleotides in order to reverse CTG expansion associated toxicity for FECD (Hu et al, 2018, 2019; Zarouchlioti et al., 2018). These studies demonstrated that antisense oligonucleotides could diminish the toxic effects associated to the CUG expansion in *TCF4* mRNA in human CEC lines (Hu et al, 2018, 2019; Zarouchlioti et al., 2018). Moreover, the delivery and uptake of antisense oligonucleotides was assessed *ex vivo* in human corneas (Hu et al., 2018) and *in vivo* using mouse models (Chau et al., 2020; Zarouchlioti et al., 2018). Nevertheless, *in vivo* functionality and reduction of the disease associated phenotype has not yet been assessed.

Antisense oligonucleotide therapies could be an elegant approach to treat FECD, nevertheless there are some key aspects that need to be taken into consideration. Namely, it is crucial to develop an efficient delivery method. The therapeutic RNA must be delivered to the back of the cornea either by topical delivery or intracameral injection without compromising its structure and the antisense oligonucleotide must be targeted and internalized by the CECs. Furthermore, an antisense oligonucleotide therapy will require life-long treatment as RNA oligonucleotides degrade quickly *in vivo*, meaning that the therapeutic agent will have to be delivered on repetitive basis. Apart from the effect on a patient's daily life, it is necessary to determine the cost-effectiveness of such therapeutic approaches. Finally, antisense oligonucleotides targeting CTG repeat could also bind to the same repeat elsewhere in the genome, potentially evoking an undesired off-target effect.

7.3. CRISPR/Cas9-based modulation

Nucleases offer the possibility to modulate genomic regions by cleaving specific targets and promote cellular responses for DNA damage repair. With the use of nucleases, genomic regions can be removed, and/or genes can be modified or inserted if a DNA template sequence is co-delivered with the desired nuclease (Moore et al., 2018). The ease of target modulation and the high specificity for sequence cleavage of clustered regularly interspaced short palindromic repeats (CRISPR)/-Cas9 system compared to other programmable nucleases such as zinc finger nucleases and meganucleases (Cox et al., 2015) positions CRISPR/Cas9 as the preferred genome editing tool to reach therapeutic use. CRISPR/Cas9 for genome editing and gene regulation has been thoroughly described elsewhere (Jiang and Doudna, 2017; Wang et al., 2016).

CRISPR/Cas9 could potentially be used to remove CTG expansions in the *TCF4* gene in order to revert the mutation causing Fuchs' endothelial corneal dystrophy regardless of the genetic mechanism causing the disease and the number of CTG repeats present. Efforts for developing CRISPR/Cas9 therapies to correct other diseases caused by trinucleotide expansion, such as Huntington's disease (Shin et al., 2016; Yang et al., 2017), paves the way for this approach.

Being a relatively new approach, studies published using CRISPR/Cas9 technology to correct genetic alterations related to corneal endothelial dystrophies are currently very limited. In 2020, Rong and colleagues demonstrated the possibility to reduce the accumulation of *TCF4* mRNA containing the CUG expansion by targeting it with an endonuclease defective Cas9, similar to an antisense oligonucleotide therapy technology known as CRISPR interference (Rong et al., 2020). In a different strategy, Uehara and coworkers demonstrated that the removal of *COL8A2* gene allele containing a missense mutation using CRISPR/-Cas9 prevented a mouse model from developing early onset Fuchs' endothelial corneal dystrophy (Uehara et al., 2020). While still in an early stage, these first studies have set the basis for the continued development of this approach.

While CRISPR/Cas9 is a promising therapeutic tool for the treatment of corneal endothelial dystrophies, it is important to highlight some of its limitations. For example, it is necessary to develop a delivery platform of both the Cas9 protein and single guide RNA, assuring that both can reach the CECs in the back of the cornea. This delivery platform should be administered topically or via an intracameral injection. Despite outperforming other nucleases in high-fidelity targeting, CRISPR/Cas9 could still cut or edit off-targets. It will be crucial to study the potential off-target effects that such therapy could generate in the cornea and eye in order to assure its safety.

7.4. General considerations

There are crucial aspects that must be considered for designing a successful genetic modulation strategy, which have been extensively reviewed elsewhere (Anguela and High, 2019), some of which need to be addressed for developing a successful genetic modulation strategy for treating corneal endothelial dystrophies. It will be important to elucidate the main genetic mechanism behind the corneal endothelial dystrophy in order to strategically design a successful therapy, as each genetic modulation strategy is best-suited to certain genetic disease mechanisms. Furthermore, defining the relationship between genotype and disease phenotype is of utmost importance. For example, there are no readily available techniques to determine the size of known trinucleotide repeats in CECs apart from gene sequencing, which is impossible to perform without a biopsy of the corneal endothelium and should be avoided due to potential tissue damage. It is crucial to develop such techniques in order to detect genetic alterations related to corneal dystrophies as well as characterizing the effect of genetic modulation therapies.

The current lack of *in vitro* and *in vivo* models for FECD also hamper

the research of genome modulation strategies. The development of *in vitro* and animal disease models presenting the genetic and phenotypic characteristics of late-onset FECD is paramount to study delivery and safety, including genotoxicity studies, but also to determine clinically meaningful end-point parameters to assess the efficacy of the selected approach.

Finally, while genetic modulation of the corneal endothelium could correct the genotype behind the disease, it will not necessarily treat existing symptomatology. For this reason, it remains to be understood when it would be most successful and feasible to treat patients, and whether it must be done before symptomatology appears, for example by performing a genetic background check on a presymptomatic patient, or at an early disease stage, when symptoms start to develop.

8. Societal challenges and ethical perspective

There is currently a global donor corneal tissue shortage, whereby only one in seventy patients worldwide have access to donor tissue for transplant. Underlying this figure is an imbalance between corneal blindness and access to corneal transplantation in different regions. Most of Western European countries, Northern American countries, Brazil, Singapore and Australia do not suffer from severe tissue donor shortage (Gain et al., 2016). In fact, countries such as the United States of America, Italy or the Netherlands are net exporters of corneal donor tissue. On the other hand, African, Asian, and some South American and Middle Eastern countries suffer from great tissue scarcity (Gain et al., 2016). The reasons behind such tissue scarcity are the lack of infrastructure that would allow cornea tissue donation, processing and storage, such as tissue banks but also cultural reasons that prevent tissue donation among citizens. It is essential to understand this unbalanced global map while developing therapies for treating corneal endothelial disease.

Corneal endothelial regenerative medicine aims to create an alternative to corneal transplantation, which would especially benefit the countries suffering from major tissue scarcity problems. Unlike Europe and the United States, where bullous keratopathy and FECD are the major indications for corneal transplantation (Gain et al., 2016), the main indication for corneal transplantation in countries suffering from major tissue scarcity are infectious keratitis and trauma (Gain et al., 2016; Matthaei et al., 2017) and these cannot be treated by corneal endothelial regenerative therapies. Nevertheless, 18% and 22% of the corneal transplantations in Africa and Asia, respectively, are still indicated for corneal endothelial disease (Matthaei et al., 2017). Alleviating this burden would liberate donor corneas for other indications.

Corneal endothelial regenerative medicine approaches also raise questions from a societal perspective. Cell and gene therapies will likely be more expensive than corneal transplantation. As a reference, the Holoclar autologous stem cell therapy for treating limbal stem cell deficiency has a selling price of USD 105,000 per eye in Europe (Shukla et al., 2019) and Voretigene neparvovec, commercially known as Luxturna, a gene therapy for correcting the defective *RPE65* gene in retinal cells has a selling price of USD 850,000 per patient in the UK (Yanuzzi and Smiddy, 2019). Cost effectiveness of CEC therapies could be addressed by the use of cell carriers. A recent analysis by Mehta and colleagues suggested the selling price of a tissue-engineered CEC graft could be comparable to a donor graft (Tan et al., 2014). From the patient perspective, at present, the only therapy for advanced disease is corneal transplantation. CEC injection could potentially address donor shortage and avoid the limitations of DMEK such as graft dislocation, and technically challenging surgery, especially in cases such as failed grafts and poor visibility. Currently, most patients are diagnosed and treated after a significant loss of CECs. Gene therapy could halt and potentially reverse the degeneration of CECs in early FECD patients, obviating the need for transplantation. In turn, FECD patients with central guttae and clear periphery could benefit from Descemet stripping only in combination with ROCK inhibitors that obviate the need for allogeneic donor tissue or long-term use of steroids and associated side effects.

Any regenerative therapies will require cGMP facilities to produce the therapeutic product and will be strictly quality controlled by the national regulatory authorities. The field of translational medicine, while it purports to "bring the bench to bedside" the reality is significantly more complicated than that in reality. After a successful clinical trial, any therapeutic product must either provide a very strong rationale for a return on investment to elicit commercial interest or face a future where it can neither be produced under the European Union Hospital Exemption, a European Union regulation foreseen in Regulation (EC) 1394/2007 with defined minimal criteria intended to provide patients the possibility to benefit from an innovative individual treatment in the absence of valid therapeutic alternatives, nor clear the hurdles of Marketing Authority. In fact, the very first ATMP to achieve marketing approval in Europe, ChondroCelect, a product composed mainly of autologous chondrocytes, has already been withdrawn from the market due to the high cost associated with its production rendering it difficult to attain reimbursement. While advances in technology and scale could reduce costs in future, regenerative therapies are only foreseeable in richer self-sufficient countries, with few exceptions of countries such as Japan, which suffers from donor shortage and has a cell therapy program. As the costs of treatment become more reasonable in the future, however, such advanced therapies will become more accessible and feasible for countries that suffer most from tissue scarcity.

The current view is that an expensive therapy in a country that is self-sufficient in donor tissue should provide an improvement upon the therapeutic outcome of the existing therapy. For a cellular or genetic therapy for corneal endothelial disease, they should therefore be benchmarked against the price and outcomes of the current DMEK. CEC therapies could allow better control of the number of live cells delivered compared to manually dissected or peeled grafts. Bioengineered grafts could be seeded with a higher CEC density ($\geq$3000 cells/mm^2), potentially increasing graft survival. Moreover, to effectively tackle the worldwide donor tissue scarcity, it is necessary to promote a global approach. If a cellular or genetic therapy is employed in a country that is self-sufficient in terms of corneal transplants, this would generate a local donor cornea surplus. Countries with major tissue scarcity would indirectly benefit from such a situation as they would be able to import sufficient corneas. Finally, affordable logistic solutions to transport bioengineered endothelial grafts and frozen CECs suspensions over long distances could allow countries lacking a GMP infrastructure to benefit from CEC therapies.

On the other hand, DSO combined with pharmacological modulation and acellular grafts present distinct advantages compared to endothelial keratoplasty, CEC, or genetic therapies. Their relatively low cost and the minimal infrastructure needed could allow their implementation, especially in countries with severe donor tissue scarcity, thereby reducing the need for corneal transplantation. Moreover, the use of regenerative approaches in self-sufficient countries could indirectly benefit countries in need by freeing donor tissue for use. Nevertheless, both DSO and acellular grafts have drawbacks compared to DMEK or cell therapies. DSO is unlikely to benefit patients with advanced FECD or bullous keratopathy, and the long-term outcomes of such interventions remain to be determined. Furthermore, in case of DSO failure, the corneal edema will worsen, and it will require access to donor corneas as only an endothelial graft will be able to improve it, a major drawback in countries suffering from tissue scarcity. Moreover, DSO is only suitable for FECD patients, and most patients in developing countries have bullous keratopathy.

Regenerative therapies should be weighed or combined with the global development of efficient eye bank infrastructures and ocular surgery facilities as well as promoting organ donations though legislation and education of populations that are reluctant to donate organs due to religious, cultural or other concerns. The current worldwide shortage of donor corneas, which is expected to increase as the population grows older, can only be tackled with a global effort and a

communal attitude.

9. Approaches for corneal endothelium regenerative medicine regulatory framework in the European Union: from bench to bedside

To make the successful translation of the therapies discussed in this review from bench to bedside, it is imperative to consider the regulatory framework and strategy at an early stage of development. In the European Union, there is specific guidance on good manufacturing practices and clinical development of therapies to assure quality and safety of the product. Depending on the approach taken for corneal endothelial regeneration, a therapy can be classified as a medical device, an advanced therapeutic medicinal products (ATMP), or a medicinal product. The different classifications have a significant impact on the regulatory path to the patient.

Medical devices are products or equipment intended for a medical use, and are generally regulated on a Member State level according to Regulation (EC) 2017/745. Acellular corneal endothelial graft substitutes or endothelial keratoprotheses would be considered medical devices, nevertheless classification boundaries might vary on a case-by-case approach. Regulation (EC) 2017/745 issued by the European Comission regulates medical devices repealing the previous Directive 93/42/EEC which regulated medical devices at national level. The European Medicines Agency (EMA) has no responsibility for the regulation of medical devices unless the medical device contains a medicinal product as an ancillary substance in which case scientific opinions are provided.

ATMPs are medicinal products based on cells or gene transfer and can be classified as cell therapeutic medicinal product (CTMP), gene therapeutic medicinal product (GTMP) or tissue engineering product (TEP). The EMA regulates ATMPs via a centralized procedure under the ATMP Regulation (EC) 1394/2007. As it currently stands, a CEC-based therapy, whether delivered as cell injection or as part of a tissue engineered corneal endothelial graft, will be categorized as an ATMP. On the other hand, genetic modulation approaches would only be categorized as ATMPs if the active substance is a biological medicinal product, meaning it is produced or extracted from a biological source. Examples for this would be gene delivery via viral vectors or plasmids. It is important to highlight that CRISPR-Cas9 based therapies would be considered ATMPs if delivered via viral or plasmid vector, but if delivered as a Cas9 recombinant protein together with a synthetic guide RNA, they would potentially fall outside the ATMP framework and could be classified as medicinal products. Nevertheless, there is no precedent for such case yet, and every case should be assessed individually. The boundaries for product classifications can change over time and should always be determined for the specific product.

A substance or a combination of substances with properties to restore, correct or modify physiological conditions in humans are considered medicinal products. The EMA regulates medicinal products under Directive 2001/83/EC. This includes recombinant proteins and pharmaceuticals that can be used for modulation of the corneal endothelium, but also synthetic oligonucleotides.

Apart from categorizing the different approaches, other regulations are needed for the translation of such therapies to the clinic. Regarding clinical trials, their implementation and conduction within the European Union is controlled by the Clinical Trials Information System (CTIS) under the Regulation (EC) 536/2014. Nevertheless, the competences for approval and supervision of clinical trials remain in hands of Member States. A manufacturing authorization issued by Member State national authorities is required for all stages of the clinical trials. And there are specific requirements that need to be addressed for ATMPs. Namely, Regulation (EC) 536/2014 establishes that investigational medicinal products (IMPs) need to comply with Good Manufacturing Practices (GMP) to assure the safety and reliability of the clinical trial. GMP manufacturing should be considered at an early pre-clinical developmental stage to enable successful transition towards the clinical setting. Furthermore, every batch of the treatment used for the clinical trial needs to be certified by a qualified person within national authorities.

Directive 2009/120/EC establishes a case by case risk-based approach when assessing the potential benefits of an ATMP therapy for market authorization. The risk analysis may consider the cell source (xenogenic, allogenic or autologous), cell proliferation capacity, cell manipulation, functionality, and preclinical and clinical data regarding functionality, safety and efficacy of a cell therapy. Directive 2010/84/EC defines a pharmacovigilance system to collect, detect, assess, and monitor possible adverse effects. Finally, directives 2004/23/EC, 2006/17/EC and 2006/86/EC provide guidance for process donation, procurement, testing, storage and traceability in case a therapy originates from human tissues.

To facilitate navigation through the regulatory framework and understand the requirements needed at every developmental stage, researchers and developers are encouraged to seek advice at an early preclinical stage as well as during later clinical development. Within the EMA, legal and regulatory scientific guidance can be enquired through the EMA innovation taskforce and the small and medium enterprise (SME) office. Moreover, the scientific advice working party of the EMA can provide scientific advice and guide researchers throughout the different steps of a therapy development process, based on a case-by-case approach. National advice can also be requested from certain member states of the European Union. The request of early guidance by researchers or developers will provide the necessary tools to understand the requirements for the approval of specific products at every development stage and allow a smooth navigation through the regulatory framework.

Finally, experience learned with the marketing approval of other ophthalmologic therapies, such as the process for marketing authorization of Holoclar (Pellegrini et al, 2016, 2018), might be valuable guidance to understand the requirements that should be met to prove safety and efficacy of a therapy so that a positive benefit–risk balance can be achieved.

10. Conclusions and future directions

Advances in protocols for the expansion of primary human CECs and their *in vivo* delivery are challenging the current one donor–one patient paradigm. It is very likely that research will develop protocols to successfully culture older donor corneas but also increase the number of CEC that can be obtained from a single donor. Furthermore, future research on the derivation of CECs from pluripotent stem cells may generate a new cell source for therapy, thereby obviating the need for allogeneic donors. A current clinical trial in Japan using iPSC–derived corneal epithelial cells for treating patients with limbal stem cell deficiency (UMIN000036539) could set the ground for future pluripotent stem cell–based therapies to treat corneal diseases.

Future results on the ongoing CEC–based clinical trials in Japan and Singapore will provide deeper understanding on the feasibility of CECs delivery methods. It is highly possible that the amount of CEC used in cell injection can be reduced compared to the currently used million cells/eye, which will allow to increase the number of patients treated from a single donor. Moreover, the ongoing clinical trials will help defining clinical endpoints for CEC therapies, highly relevant for regenerative medicine therapies as they are an integral part of the regulatory approval (Schlereth et al., 2021). Finally, synthetic CEC carriers would enable a more cost–effective, limitless and standardized alternative.

Acellular corneal endothelial grafts provide an alternative therapeutic approach that will be dramatically cheaper, and especially beneficial in developing countries. Future clinical trials will help to understand how long a cornea can be kept transparent using such devices.

Lamellar corneal transplantation is presently the default procedure to treat corneal endothelial disease. With an increase in the available therapeutic arsenal, it is critical to select the best treatment option for each patient. In the coming years, research will allow deeper understanding of what spectrums of corneal endothelial disease could be successfully treated with each approach. It is possible that young patients with long CTG repeats in the *TCF4* gene and early to moderate FECD could be candidates for genetic modulation. On the other hand, FECD patients with a good peripheral corneal endothelium could be treated with ROCK inhibitors alone or in combination with DSO/DWEK. Severe bullous keratopathy and advanced FECD could be treated by lamellar keratoplasty or cell therapy delivered by injection for bullous keratopathy cases or using a carrier for FECD cases. A personalized medicine approach will allow greater access for more people to therapy and tackle global donor shortage.

Author statement

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