Transplantation of cultured corneal endothelial cells: Towards clinical application

Spinozzi D.

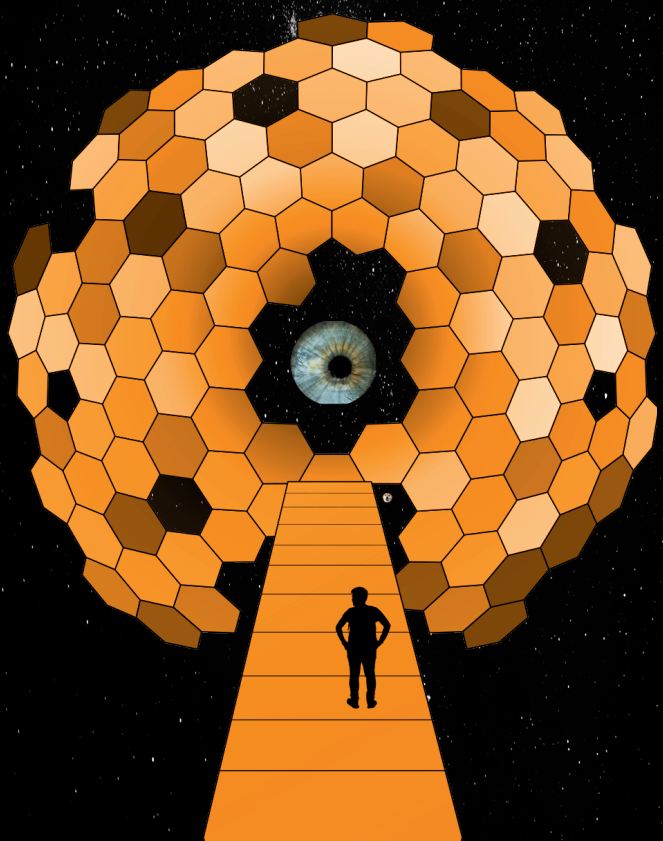
CHAPTER 2

New developments targeting corneal endothelial cell replacement.

Daniele Spinozzi[1], Alina Miron[1], Marieke Bruinsma[1], Isabel Dapena[1,2], Viridiana Kocaba[1,2,3], Martine J. Jager[4], Gerrit R.J. Melles[1,2,5], Sorcha Ní Dhubhghaill[1,2,6], Silke Oellerich[1]

[1]Netherlands Institute for Innovative Ocular Surgery, Rotterdam, The Netherlands; [2]Melles Cornea Clinic Rotterdam, The Netherlands; [3]Tissue Engineering and Stem Cell Group, Singapore Eye Research Institute, Singapore; [4]Dept. of Ophthalmology, Leiden University Medical Center, Leiden, The Netherlands; [5]Amnitrans EyeBank Rotterdam, The Netherlands; [6]Antwerp University Hospital (UZA), Edegem, Belgium.

Review. Submitted for publication.



ABSTRACT

Corneal transplantation is currently the most effective treatment to restore corneal clarity in patients with endothelial disorders. Endothelial transplantation, either by Descemet Membrane Endothelial Keratoplasty (DMEK) or Descemet Stripping (Automated) Endothelial Keratoplasty (DS(A)EK), is a surgical approach that replaces diseased Descemet membrane and endothelium with tissue from a healthy donor eye. Its application, however, is limited by the availability of healthy donor tissue. To increase the pool of endothelial grafts, research has focused on developing new treatment options as alternatives to conventional corneal transplantation. These treatment options can be considered as either "surgery-based", i.e. tissue-efficient modifications of the currently endothelial keratoplasty techniques (e.g. Descemet Stripping Only (DSO)/Descemetorhexis Without Endothelial Keratoplasty (DWEK), hemi- and Quarter-DMEK), or "cell-based" approaches, which rely on *in vitro* expansion of human corneal endothelial cells (hCEC) (i.e. cultured corneal endothelial cell sheet transplantation and cell injection).

In this review, we will focus on the most recent developments in the field of the "cell-based" approaches. Starting with the description of aspects involved in the isolation of hCEC from donor tissue, we then describe the different natural and bioengineered carriers currently used in endothelial cell sheet transplantation, and finally we discuss the current "state of the art" in novel therapeutic approaches such as endothelial cell injection.

KEYWORDS: cornea, corneal endothelium, transplantation, tissue engineering.

INTRODUCTION

The corneal endothelium is a monolayer of hexagonal, tightly packed cells that marks the posterior part of the cornea. Human corneal endothelial cells (hCEC) are not thought to be capable of replication *in vivo*, being held in the G1 phase of the cell cycle.[1-3] As a result, hCEC have a restricted lifespan, meaning that their density and number decline naturally with age, with a decrease of ~0.6% per year from about 3500 – 4000 cells/mm^2 at birth to 2300 cells/mm^2 by the age of 85.[4-6] When hCEC are damaged, a cascade of events are initiated to repair the defect. Adjacent cells migrate centripetally, form new tight junctions and finally restoring the cellular pump function. During this process, the migrating cells remodel from an irregular endothelial cell to a more hexagonal pattern, though with fewer cells/mm^2.[7] The minimum density reported for a corneal endothelium to remain functional is thought to be approximately 500 cells/mm^2.[8]

Two of the most common pathologies of the corneal endothelium are Fuchs endothelial corneal dystrophy (FECD)[9] and bullous keratopathy.[10] These pathologies are commonly treated by corneal transplantation to restore corneal clarity in patients with endothelial disorders.[8] Descemet membrane endothelial keratoplasty (DMEK) is the most selective corneal transplantation technique, where the patient's Descemet membrane (DM) and damaged endothelium are removed and replaced with the same layers, from a donor eye.[11,12] However, its application is limited by global shortage of high-quality donor tissue, with only one donor available for every 70 patients in need of a transplant.[13] Different strategies have been applied in the past decades to solve the tissue shortage and these may be broadly separated into two approaches: the surgical and the "cell-based" approach.

Surgical approaches to target tissue shortage include Descemet Stripping Only (DSO)/Descemetorhexis Without Endothelial Keratoplasty (DWEK) and Quarter-DMEK technique, which were developed for eyes with central FECD. With DSO/DWEK, the diseased tissue is removed without replacement by donor tissue. The stripped region is re-populated by the migration of the peripheral endothelial cells that can restore corneal clarity, though with varying rates of success.[14-16] Quarter-DMEK resembles standard DMEK, but the donor

DM-endothelial cell sheet is divided into four grafts allowing a much more efficient use of donor tissue.[17,18] Clinical results up to 2-year follow-up for Quarter-DMEK show visual rehabilitation comparable to standard DMEK, but a higher endothelial cell density (ECD) decrease than standard DMEK.[19,20]

The "cell-based" approaches rely on the *in vitro* expansion of isolated primary hCEC (**Figure 1**), as hCEC have been shown to be able to proliferate *in vitro* if given the appropriate stimuli.[21] Currently, the main strategies to deliver hCEC onto the posterior corneal surface are cultured endothelial cell sheet transplantation and cell injection into the anterior chamber. Endothelial cell sheet transplantation requires a suitable cell carrier, which can have a natural origin or be a bioengineered matrix, and is currently still in a preclinical stage. Cell injection therapy, on the other hand, obviates the need for a cell carrier, as the cultured hCEC are injected directly into the patient's anterior chamber. For this approach, first clinical results have been reported.[22,23]

In this review, we focus on the two "cell-based" approaches, cell sheet transplantation and cell injection, by covering the entire approach from *in vitro* growth of primary hCEC over potential carrier for cell sheet transplantation to considerations for cell injection therapy to first pre-clinical and clinical result.

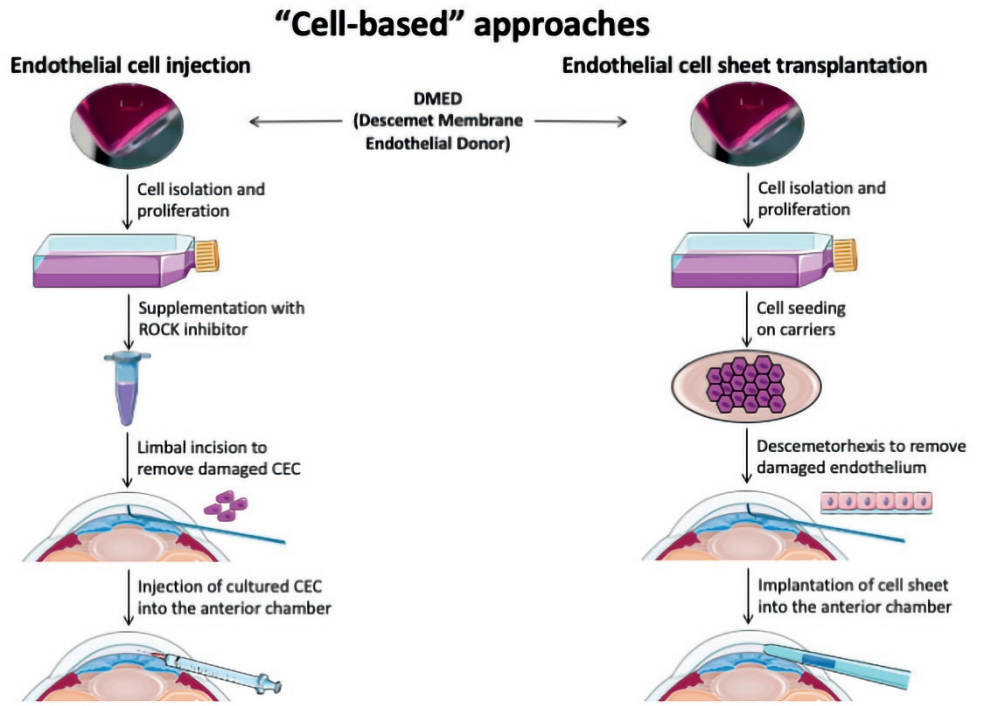


Figure 1: "Cell-based" approaches. Schematic representation of the two "cell-based" approaches described in this review: the endothelial cell injection approach and endothelial cell sheet transplantation approach. They both rely on the availability of a donor tissue (DMED = Descemet Membrane Endothelial Donor), from which hCEC will be isolated and subsequently cultured and expanded in vitro. At this point, for the cell injection approach, suspended hCEC will be supplemented with ROCK inhibitor and injected into the anterior chamber of the patient, after having removed the damaged endothelial tissue from the eye of the patient. For the endothelial cell sheet transplantation approach, cultured hCEC are passaged upon confluency, suspended in culture medium, seeded on the desired carriers, and then transplanted following a DMEK-like surgical protocol, upon descemethorexis to remove the damaged endothelial layer.

IN VITRO CULTURE AND EXPANSION OF CORNEAL ENDOTHELIAL CELLS

Although hCEC have limited proliferative capacity *in vivo*, they do have the ability to proliferate *in vitro*. After the first reported successful *in vitro* expansion of hCEC in 1965,[24] a plethora of protocols describing the isolation and *in vitro* growth of hCEC have been published.[8,23,25-49] The abundance of protocols is mainly due to the challenges inherent in culturing and expanding hCEC *in vitro*, and so far, no protocol appears to be superior regarding the consistent expansion of hCEC. Successfully establishing a robust and reproducible cell culture protocol for hCEC isolation and expansion requires a fine-tuning of all the procedures involved: selection of suitable donor tissue, peeling of the corneal endothelium and DM from donor corneas, enzymatic digestion to isolate the hCEC, seeding of the resulting cell suspension using a combination of culture media and growth factors, and expansion and proliferation on appropriate substrates that mimic the *in vivo* conditions. It has been shown that the induced loss of cell contacts and the supplement of the culture medium with selected growth factors boost hCEC growth.[50] Multiple extracellular signals, however, can activate critical intracellular pathways and induce endothelial-to-mesenchymal transition (EMT) of hCEC, where the cells acquire a fibroblast-like phenotype thereby losing their morphological features and, most importantly, their function.[8,34,40,50,51] Therefore, a lot of effort has been put in designing the appropriate protocols for hCEC isolation, expansion and propagation with a focus on maintaining hCEC morphology and function.

Sources for corneal endothelial cell culture

Primary hCEC isolated from cadaver donor corneas, stem cells, and cell lines have all been used as sources for corneal endothelial cell culture.[52] The main sources of primary hCEC are human cadaveric donors, however, donor characteristics such as cell density,[41] cause of death, previous surgery in the donor eye,[53] overall health of the donor, and tissue storage time can have a significant impact on the culture success rate.[54,55] Donor age can also play a role[42] with a

usually lower proliferation capacity for hCEC derived from older donors.[25,29,33,34,45,47,56-61]

It has also been reported that hCEC from the corneal periphery have a higher proliferation capacity than hCEC from the corneal center,[30,55,57,58,62,63] though another study showed no difference in the replicative capacity between the peripheral and central areas of the cornea.[55] Attempts to overcome the lack of donor material from which to isolate and expand hCEC *in vitro*, have included the immortalization of CEC via viral transfection,[64,65] the disruption of the balance of cell cycle regulators,[66,67] and the development of genetic transformations that resulted in immortalized hCEC lines.[68]

Stem cells used for hCEC culture include organ-specific adult stem cells obtained from adipose tissue, umbilical cord blood or bone marrow,[69-71] directed differentiation competent embryonic stem (ES) cells,[72] induced pluripotent stem (iPS) cells,[73] and hCEC precursors.[74] For the latter, it has been suggested that existent stem-like progenitors of hCEC may retain more proliferative capacity compared to terminally differentiated hCEC.[75] It is thought that such hCEC progenitors could be present in the area that separates the peripheral endothelium from the anterior part of the trabecular meshwork, and they might act as a cell supply activated in the event of a wound healing.[35,37,74,76-81] However, these cells have been hard to identify because of a lack of suitable markers and their existence is still debated.[77] In addition, the amount of stem cells found in this compartment of the cornea may be very low and could require an extended *ex vivo* culture to generate sufficient cells for transplantation.[82]

Methods of isolation of corneal endothelial tissue

Currently, the most used protocol for cell isolation consists of a two-step peel-and-digest method (**Figure 2**).[34,35,38,39,42,43,45,47,54,60,83-93] The Descemet Membrane Endothelial Donor (DMED) is first peeled from the underlying stroma of the donor cornea, followed by dissociation of cell junctions to separate the hCEC from the membrane. In respect to the method used to disrupt cell junctions, the tissue digestion strategies may be classified as enzymatic and non-enzymatic.[82] The enzymatic digestion procedures are based on enzymatic treatments such as

collagenase, trypsin, or dispase, while the non-enzymatic tissue digestion uses ethylenediamine tetraacetic acid (EDTA) to release cell-cell junctions.[60] In enzymatic digestion, several approaches have been proposed,[26,51] including the use of collagenase A as this enzyme induces a careful, selective reduction of the intercellular matrix with minimal damage to cell membranes and the ability of hCEC to expand.[35,38,74] Another enzymatic digestion approach uses trypsin, which has been used successfully to cultivate and expand hCEC on bovine extracellular matrices.[29] Nevertheless, trypsin mainly acts on the intracellular mucoproteins, thus affecting the cell membrane and is typically used in passaging monolayer cultures. For a more effective action during cell isolation, trypsin is often used in combination with EDTA, which disrupts lateral cell contacts thus separating cells from each other as well as from the culture surface without negative effects on cell viability.[94] Liberase and dispase have also been shown to be useful in the isolation of CEC from corneal endothelial tissue.[49,83]

The non-enzymatic method is based on the mechanism of action of EDTA to release cell junctions while it enhances cell division upon exposure to mitogens.[54,55,62,70,94] Other approaches, usually applied in the beginning of hCEC isolation, include hCEC scraping from the DM,[27] enzymatic digestion of hCEC on the cornea,[26] a combination of enzymatic digestion of hCEC on the cornea and scraping,[55] and explant culture.[28,33,42,95-99] Furthermore, recent efforts have been made to isolate a purer population of hCEC with high proliferative capacity by density-gradient centrifugation to eliminate senescent cells.[100] However, because of different issues of both enzymatic and non-enzymatic methods alone (contamination with other cellular types and decrease of cellular yield, respectively), the preferred method to date is a combined treatment that includes collagenase, to derive hCEC aggregates from the tissue, and incubation for short time with trypsin/EDTA to produce less cell damage.[82]

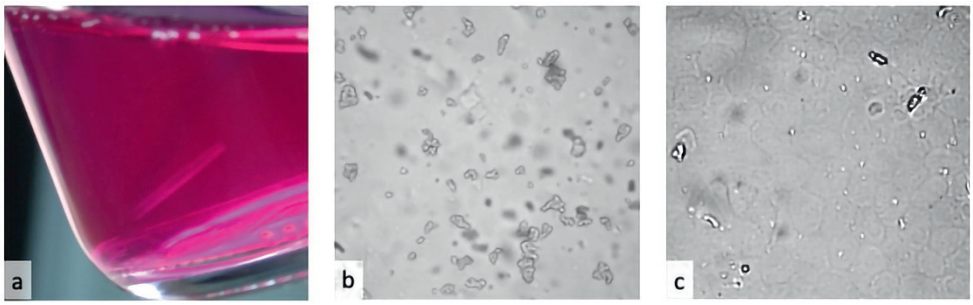


Figure 2: The "peel-and-digest" method. Series of images illustrating the 2-step, "peel-and-digest" method applied for the hCEC isolation from donor tissue. After the peeling of the DMED from the donor cornea, the tissue is stored accordingly in the eye bank (a). Upon hCEC isolation, DM-EC sheet is exposed to collagenase digestion for 4-6 hours to disrupt the cell junctions and to obtain smaller fragments, which will be further dislodged in single cell suspension by flushing and centrifugation (b). After 2 weeks in culture, hCEC were able to form a uniform monolayer of tightly packed cells, which retained their characteristic hexagonal morphology (c).

Culture media and growth factors

Many hCEC culture media have been described with a combination of different base media (e.g. M199, L-valine-free IF, F99, Ham's F12 + M199, Ham's F12+DMEM (SHEM), MEM, Endothelial growth medium, DMEM, OPTIMEM-I), essential and non-essential amino acids (e.g. glutamine), sera (e.g. bovine, calf, horse, human), antibiotics (e.g. penicillin, streptomycin, gentamycin, amphotericin B, fungizone, doxycycline), growth factors or a combination thereof (e.g. basic fibroblast growth factor (bFGF), insulin, nerve growth factor, bovine pituitary extract, epidermal growth factor, transferrin), vitamins (e.g. ascorbic acid, l-ascorbic acid 2-phosphate) and other components (e.g. chondroitin sulphate, calcium chloride, DMSO, hydrocortisone, cholera toxin, selenium, sodium selenite, human lipids, RPMI-1640 multiple vitamin solution, type-I collagenase, B-27, Rho-associated protein kinase (ROCK) - inhibitor, cysteine, TGFβ-inhibitor).[28,29,33,34,37-39,41,43,45,47,51,52,55,56,60,62,84-88,91,92,95,98,99]

However, while none of these media may be superior in the reproducible cultivation and expansion of hCEC *in vitro*, the use of a so-called "dual-media" approach has gained popularity.[21,42,43,89,101,102] Peh et al. were the first to describe

a dual-media approach, in which isolated hCEC were propagated using a culture system that included a proliferative medium, rich in growth factors, and a serum-supplemented culture medium, without growth factors, in order to avoid epithelial-mesenchymal transition (EMT). A modified dual-media approach has also been described to increase the rate of obtaining successful cultures from elderly donor corneas.[47]

In addition, several studies reported the use of conditioned medium from other cell types to promote hCEC growth. These include conditioned medium from murine embryonic stem cells (mESC),[39,98] human bone marrow-derived mesenchymal stem cells (hBM-MSC),[39] and human amniotic fluid (hAF).[103] The first two treatments showed to be effective in hCEC proliferation and motility by predominantly acting on the cell cycle level, while hAF-containing culture medium stimulated hCEC growth. While conditioned medium from mESC is not suitable for clinical application due to the presence of a xenoantigen for human cells, condition media derived from both hBM-MSC and hAF can be used in the clinical setting. Condition medium from hAF is cheaper and more readily available than other growth factors, however the potential interdonor variability does not make this product fully suitable for clinical applications.

Preventing endothelial-to-mesenchymal transition of hCEC during culture

Preventing EMT is one of the most challenging issues in hCEC culture.[8,34,40,50,51] During EMT, hCEC loosen their specific markers and adopt mesenchymal characteristics. While EMT actually plays an important role in normal physiological processes such as wound healing, it also interferes with pathological processes such as fibrosis.[50] The onset of EMT can be identified by typical events such as: disruption of cell-cell junctions, loss of cell polarity, modifications in cell shape and cytoskeletal organization, increment in the secretion of ECM proteins and genotypical changes (**Figure 3**).[50] This loss of endothelial phenotype represents a big limit for the use of cultured hCEC in tissue engineering applied to corneal regeneration.[40,50,104-110] Thus, in order for cultured hCEC to be applied in clinical practice, EMT must be suppressed during the culture process.[50]

Several strategies have been proposed to block EMT. As *in vitro* hCEC culture and expansion requires steps like cell isolation and passaging that inevitably cause the dissociation of cell junctions, culture conditions should be such that cell confluency and polarity are gained as fast as possible.[50] This can be accomplished by steps as simple as increasing cell seeding density.[41] During EMT, reorganization of the cytoskeleton can be visualized by the presence of α-smooth muscle actin (α-SMA).[104,111,112] The expression of α-SMA by hCEC has been shown to be promoted by transforming growth factor beta (TGF-β), a cytokine known to induce EMT.[40,104]

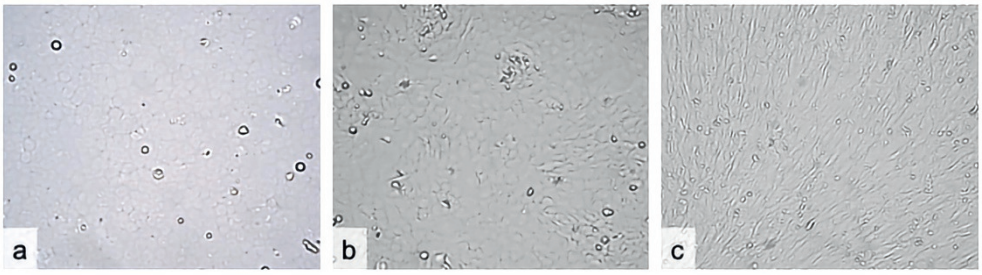


Figure 3: EMT transition in cultured hCEC. Light microscopy images showing the transition from a successful hCEC culture to a fibroblastic culture following EMT. After enzymatic digestion, hCEC are expanded and form a confluent monolayer of small, hexagonal cells (a). Due to characteristic events such as changes in cellular gene expression and phenotype and loss of cell-cell junctions, cultured hCEC could undergo EMT, facing different phases from a culture of mixed phenotype (b) to a full fibroblast-like culture (c). This process is particularly disadvantageous because it limits the use of cultured hCEC for further purposes in tissue engineering.

In normal cells, TGF-β arrests the cell cycle at the G1 stage, inhibiting proliferation and inducing differentiation. Studies have shown that hCEC are amongst the cells most sensitive to stimulation by TGF-β, with TGF-β having both pro-migratory and anti-proliferative effects on hCEC.[105] In addition, blocking the TGF-β(2) receptor in proliferating hCEC by anti-TGF-β inhibitor SB-431542 allows expansion and proliferation, by avoiding EMT and uncontrolled growth of other cell types.[40] L-ascorbic acid 2-phosphate (Asc-2P), a phosphate derivative of vitamin C,[113] acts as an antioxidant and a stimulator of

hepatocyte growth factor (HGF) production previously known for stimulation of the barrier function in endothelial cell monolayers[114] and the proliferation and replication of a number of cell types.[115,116] Adding Asc-2P to hCEC culture media can also aid in cell growth, proliferation, and in delaying EMT.[87,117]

Additionally, cells in which EMT is induced can increase the production of ECM proteins, such as collagens, cadherins and fibronectins.[40] *In vitro* models of endothelial diseases were used to find out several factors involved in EMT that induced atypical ECM secretion and fibrosis by hCEC, including FGF-2.[50] Paradoxically, FGF-2 is often used to stimulate hCEC proliferation *in vitro*, despite the risk of inducing EMT of hCEC. The fine balance between FGF-2 ability to either induce proliferation or EMT in cultured hCEC is currently under investigation.[50]

Coatings

Descemet membrane is the basement membrane secreted by the corneal endothelium and includes many proteins, such as fibronectin, laminin, collagen type IV and VIII and proteoglycans,[118-123] and considerable developments have been done towards the design of a resembling native ECM environment for hCEC growth and proliferation.[124-133]

Although hCEC are capable of attaching to and expanding on the tissue culture plates,[42,60,85,98] the use of coating materials has shown superior results. Tested types of coating materials for hCEC expansion include varieties of collagen,[31,42,51,83,85,87,92,96,99,134-138] fibronectin,[27,29,42,51,85,99,131,137] laminin,[51,90,99,131] and gelatin.[27,139-142] Also mixtures of different components have been used,[26,29,33,51,95-97,99,143] of which the mixture of fibronectin, collagen, and albumin is the most widely used coating (FNC coating mix®).[34,39,42,43,45,47,54,88,93,131]

It has been reported that FNC-coated culture plates improve the spreading of hCEC in culture and also reduce cell loss after rinsing.[36] Moreover, hCEC cultures grown on FNC-coated plates showed a higher success rate in terms of cell confluence and morphology compared to cultures grown on fibronectin-coated plates, collagen IV-coated plates, and uncoated plates.[42]

Laminin is another major component of the basement membrane, and is responsible for the regulation of cell migration, proliferation and differentiation.[144,145] Laminin isoform laminin-5 (LM-5) has been shown to have a positive effect on hCEC adhesion and migration.[99] Some other isoforms of laminin (laminin-511 and laminin-521), also expressed in DM, are good substrate candidates for *in vitro* hCEC culture, helping to increase cell adhesion and proliferation.[90] Moreover, the inclusion of laminin E8 fragments (truncated proteins that include the active binding site)[146] help cultured hCEC retain a better endothelial cell morphology compared to hCEC cultured on FNC coating mix.[90]

Overall, however, the biological activity of hCEC on these coatings, even among studies using the same coating material, varies. This might be explained by the type of coating affecting hCEC adhesion, morphology, proliferation, and function.[52] In addition, the variation among studies using the same coating might be explained by differences in the applied cell culture protocol.[52] Nevertheless, the translation of these coatings to the clinical application is still doubtful since they are derived from animals which may bare the risk of pathogen transfer. The development of a recombinant form of laminin E8, however, offers the possibility of a xeno-free, good manufacturing practice (GMP)-compliant substrate.[146] An alternative xeno-free substrate could be a pericellular matrix of decidua-derived mesenchymal cells (PCM-DM). This matrix has already been tested, with good results, as a substrate for human ES cells,[147] iPS cells,[148,149] and hCEC.[137]

CULTURED ENDOTHELIAL CELL SHEET TRANSPLANTATION

Human CEC, successfully cultured and expanded *in vitro,* form intercellular junctions creating a cell sheet. A cell sheet, however, is too fragile to be handled surgically and requires support. To that end, substrates or carriers that provide mechanical support during transplantation of *in vitro*-cultured hCEC have been developed and tested. An ideal carrier should mimic the basic characteristics of the DM and therefore be transparent, permeable, not too thin to provide sufficient mechanical strength (as the elastic modulus could have an impact on cell phenotype),[132] flexible to adjust to the corneal curvature, biocompatible, promoting hCEC-carrier interactions, and allowing interaction between the cultured hCEC layer and the recipient stroma in terms of exchange of nutrients and small molecules, and finally, be easily reproducible.[52] Potential carriers, that can either be natural tissue materials such as amniotic membrane, human anterior lens capsules (HALC), and decellularized DM or stroma,[46,48,68,83,84,136,143,150-153] or polymeric materials (natural and synthetic), have been explored as carriers to facilitate hCEC transplantation.[31,85,86,88,131,132,142,154-159]

Natural tissue carriers

Denuded DM and Devitalized Stroma

As DM is part of endothelial grafts, and given that devitalized corneas or denuded DM supply the desired shape, mechanical strength and transparency, they have been widely tested as carriers for hCEC.[52,60,95,130,131,143,150,152,160-162] The endothelial cells from donor DM can be removed by enzymatic treatment, in most cases with a combination of trypsin and EDTA (**Figure 4 c, d**), or via mechanical treatment or by applying several freeze/thaw cycles, to obtain a denuded DM.[95,150,160-165]

Mimura et al. showed the feasibility of corneal reconstruction with cultured hCEC seeded on denuded DM and transplanted in nude rats.[150] Up to one month after transplantation, hCEC functioned well and maintained corneal transparency. Another study, that used devitalized posterior corneal stromal

lamellae as a hCEC substrate, showed that the graft had intact barrier function and the hCEC expressed typical markers.[152] Recently, in a rabbit model of bullous keratopathy, Peh et al. showed that hCEC seeded onto denuded DM or stroma were able to induce corneal clearance.[46] However, when using devitalized corneal stroma, resident viable keratocytes may cause fibroblastic contamination.[52]

Using an *in vitro* surgery model (**Figure 5 e-h**), hCEC-denuded DM constructs behaved similarly to the DMEK reference model. The constructs, however, displayed a 'reversed' rolling with the hCEC on the inside.[166] In addition, this study showed that denuded DM derived from elderly donors show a highly structured surface due to imprints left by the removed cells (**Figure 4 c, d**). This structured surface seemed to be responsible for the impaired morphology of the cultured cells on these carriers.

Human anterior lens capsule

The human anterior lens capsule (HALC) is a transparent membrane that is mainly composed of interacting networks of laminin[167-170] and collagen type IV,[168,171,172] the latter being HALC's most prominent protein,[172] forming up to 40% of its weight.[173] In addition to its composition, the physical characteristics of HALC are also very similar to the DM in terms of thickness, elasticity and transparency.[174,175]

HALC has been investigated as a scaffold for culturing and transplanting different ocular cells,[176-180] including hCEC.[84,136,166] For these purposes, HALC can be either manually isolated from donor eyes followed by an enzymatic treatment to remove cellular layers from the surface (**Figure 4 a, b**), or can be obtained during cataract surgery, limiting on one side the available diameter to 6 mm approximately but allowing the isolation from the patients themselves.[84,136,180] It has been shown that hCEC density and morphology when grown on HALC resembled those of healthy corneas.[84] The surgical potential of CEC-HALC carrier constructs was tested in an *in vitro* surgery model (**Figure 5 a-d**).[166,180] Upon *in vitro* surgery, hCEC-HALC constructs behaved in a manner similar to the DMEK reference model. Interestingly, the hCEC-HALC

constructs exhibited a 'reversed' rolling property, with the endothelial cells on the inside. First *in vivo* tests in an animal model of CEC-HALC constructs were not successful due to the choice of the animal model system. The Goettingen mini-pigs used in that study showed an enhanced retrocorneal membrane formation limiting the information to be obtained on the CEC-HALC constructs.[181]

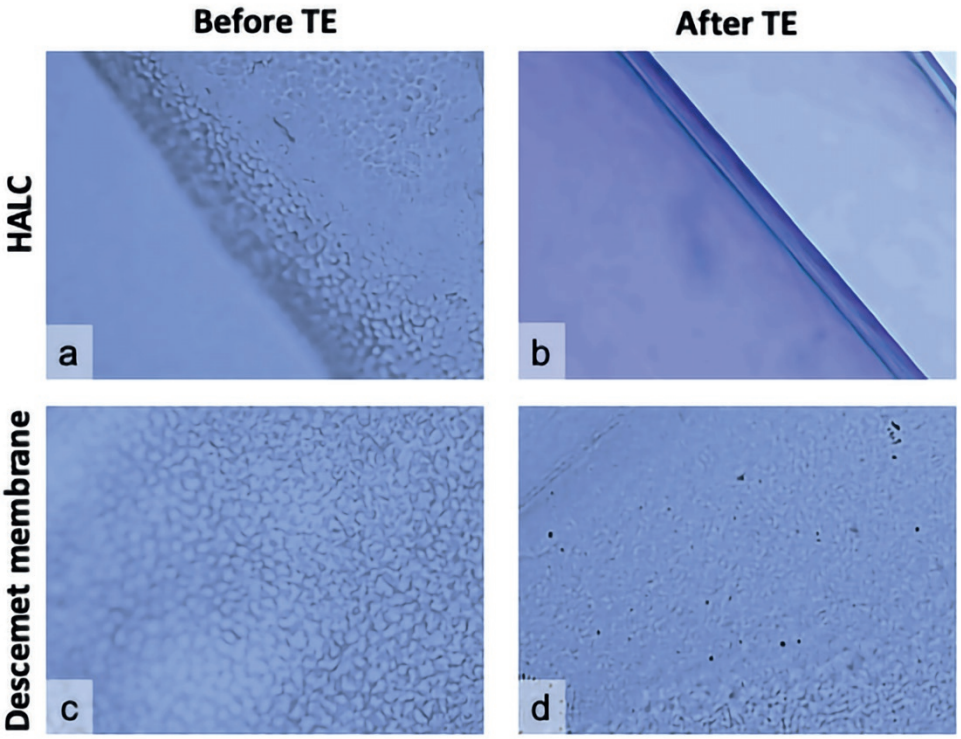


Figure 4: HALC and "denuded" Descemet membrane preparation. To use human anterior lens capsule (HALC) and Descemet membrane as a carrier for hCEC sheet transplantation, cellular layers had to be removed from both sides of the substrates. When HALC was subjected to an enzymatic treatment with Trypsin/EDTA (TE) solution for 15 minutes, smooth sides were obtained (a, b). On the other hand, the treatment of Descemet membrane with TE solution was efficient to remove the cell layer, but the surface showed a structured pattern, most likely left by the removed cells (c, d).

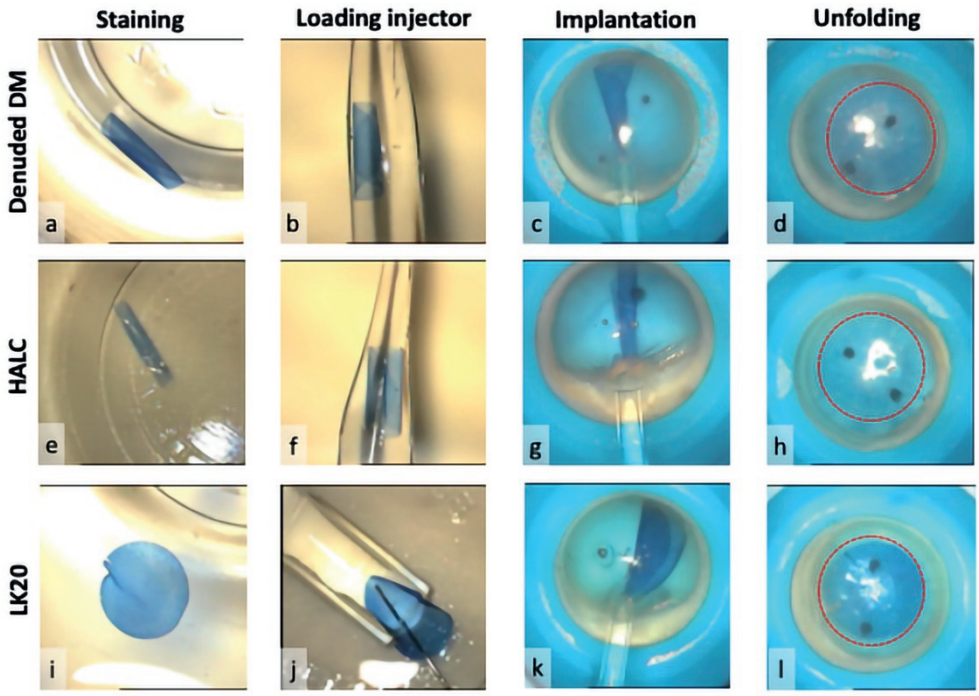


Figure 5: *In vitro* surgeries with hCEC-carrier constructs for endothelial cell sheet transplantation. *In vitro* surgeries were performed to test the feasibility of hCEC-carrier constructs as potential alternatives to corneal grafts. The analyzed carriers were Descemet membrane (DM) from which the endothelial layer was removed (denuded DM), human anterior lens capsule (HALC), and collagen I-based carrier of 20 µm of thickness (LK20). The constructs were implanted using a DMEK surgical technique into an anterior remnant without endothelium mounted onto an artificial anterior chamber and several parameters were evaluated, such as staining with hypotonic Trypan Blue solution 0.04% (a, e, i), loading into the glass injector (b, f, j), implantation into the artificial chamber (c, g, k), and unfolding (d, h, l).

Amniotic membrane

Amniotic membrane is composed of collagen type IV[182] and has been widely used for surgical purposes.[183] It was the first successful natural carrier for ocular cell culture and transplantation[184] and is known to have anti-inflammatory[185] and non-immunogenic[186] properties. It has been demonstrated by Ishino et al. that ECD and morphology of hCEC cultured on amniotic membrane were similar to those of normal corneas, and such cultured cells were also functional *in vivo*.[83] After transplantation of the hCEC-amniotic membrane constructs into rabbit

eyes, corneal edema was observed after seven days.[83] Fan et al. showed that hCEC cultured on amniotic membrane retained normal morphology and marker expression. Moreover, after transplantation in a cat model where the cornea was denuded of endothelium and DM, corneas remained transparent throughout the monitoring period.[68] However, while amniotic membrane is biocompatible along with anti-inflammatory and non-immunogenic properties, potential disadvantages are the lack of transparency, and the time-consuming preparation time.[52,187]

Bioengineered carriers

To reduce dependency on donor tissue, there is an increasing interest in tissue-engineering of (bio)synthetic cell carriers,[140,142,188,189] though the issues of biotoxicity and bio-incompatibility make this approach more challenging.[190] Ideally, the carrier should mimic the natural basement membrane as much as possible in terms of composition, architectural durability, biocompatibility with cultured CEC, transparency and compliance for clinical application.[159] Several biomaterials, both natural and synthetic polymers, have been investigated.[31,85,86,88,131,132,142,154-159] Natural polymers, such as collagen, gelatin, and silk are usually biocompatible, though synthetic polymers guarantee a fine customization of the desired properties.

Collagen-based carriers

Collagen is the most abundant component of ECM in most tissues.[159] While it is easy to produce, cheap to derive from animal sources, and compatible with corneal cells, by itself it is not sufficiently rigid to handle surgically. Crosslinking the collagen improves its mechanical strength and chemical stability, and this can be achieved by chemical, physical, and enzymatic procedures.[159] One concern about the use of crosslinked collagen is the generation of toxic residues.[191]

Culturing hCEC on collagen sheets is not new, as studies in this area date back to the 1980s,[192] and showed promising results in animal studies.[92,96,193] Mimura et al. demonstrated that cultured hCEC, transplanted via a crosslinked collagen-1

sheet, can maintain corneal dehydration in a rabbit model.[96] When hCEC were cultured on collagen-vitrigel, they displayed a strong expression of ZO-1 and Na^+/K^+-ATPase, and reduced corneal thickness and improved corneal transparency in a rabbit model.[193] In another rabbit study, transplanted hCEC-collagen hydrogel sheets maintained a high cell density and N-cadherin, ZO-1, and actin-filament displayed a similar expression pattern as in native hCEC.[92]

While these studies reveal that cells can maintain their morphology and function on the collagen, the flexibility and thickness are also important to consider. Collagen type I-based carriers, of different thickness, have been tested by *in vitro* surgery. A carrier of 20 μm thickness (LK20) exhibited good biocompatibility, however, during *in vitro* surgery, the hCEC-LK20 constructs displayed poorer adherence to the posterior stroma than the other carriers tested (**Figure 5**).[166] A carrier of 100 μm thickness of the same material was found too rigid for the intended surgical procedure.[180]

Modifying the collagen to create collagen-polymer composites (chitosan, chondroitin sulphate) has been reported to further increase the mechanical support and resistance to enzymatic degradation.[159] Plastic compressed collagen type I, termed Real Architecture For 3D Tissues (RAFT) may offer better mechanical properties when compared to crosslinked collagen.[88] The formation of a confluent monolayer of hCEC which expressed ZO-1 and Na^+/K^+-ATPase on these carriers was confirmed by scanning and electron microscopy imaging.

Although the deep understanding of collagen-based sheets as a potential carrier for transplantation, the translation to clinical application still faces several challenges. Collagen is a material derived from animal origin and this raises questions in terms of immunogenicity and interspecies transmission of diseases.[194] Moreover, the extraction and purification procedures make collagen very expensive for large amounts of material needed for clinical applications and such procedures can vary from company to company, resulting in unwanted batch-tobatch variations.[195] Clinical-grade carriers should guarantee cell adherence and pumping function for the cells seeded on the carriers (**Figure 6**). Several research groups have tried to synthetize recombinant human collagen, but the low yield and the scarce post-translational modifications still represent unsolved issues.[196]

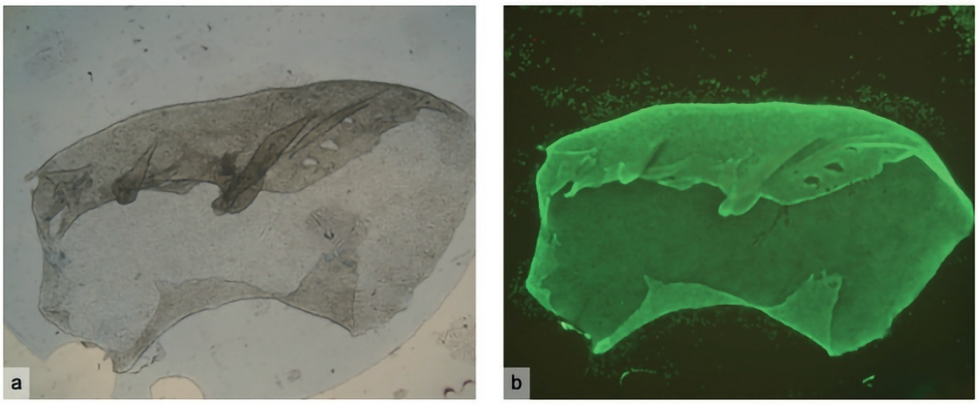


Figure 6: Cultured hCEC adherence on collagen-based carriers. After hCEC seeding on a collagen-based carrier, the cell-carrier construct was tested for its feasibility as an alternative to endothelial graft upon *in vitro* surgery. After the surgery, the cell layer detached almost completely from the carrier below (a), although the Calcein-AM staining confirmed that the seeded hCEC were viable (b). These results show that cell adherence still represents a parameter that should not be taken for granted in the development of a firm interaction between cultured hCEC and biocompatible carriers.

Gelatin-based hydrogels

Gelatin is a commonly obtainable natural material originated from the hydrolysis of collagen,[159] and when crosslinked, gelatin-based cell carriers can be used for tissue-engineering.[159] Watanabe et al., showed that primary hCEC seeded on hydrothermally crosslinked gelatin could form a confluent monolayer and showed normal marker expression.[142] Adding heparin during chemical crosslinking produced a gelatin carrier flexible enough to be folded, and that maintains morphology and pump function of hCEC seeded onto it.[156] The use of semisynthetic gelatin methacrylate registered an increase in tissue engineering because of its biocompatibility and adaptable physical characteristics,[159] and has been shown to support hCEC *in vitro* as well as after transplantation into a rabbit model.[158]

Chitosan

Chitosan is a biomimetic polysaccharide derived from the deacetylation of chitin.[188,189] Blended membranes made of chitosan, gelatin and chondroitin sulfate have displayed biomechanical properties (e.g. transparency, ion and glucose permeability) comparable to the human cornea. During *in vivo* testing in rabbit eyes, the CEC-blended membrane constructs showed steady degradation of the membrane and this did not influence the structure and the curvature of the cornea.[197] A keratin-chitosan membrane has also been shown to support CEC attachment and growth while maintaining cell morphology and marker expression.[198] In addition to providing a carrier for hCEC, the incorporating chitosan can improve the optical transparency and mechanical support of collagen membranes.[197] In another study, a chitosan-based membrane was constructed of hydroxypropyl chitosan, gelatin, and chondroitin sulphate.[199] Although optical transparency was comparable to the natural human cornea and cultured rabbit corneal endothelial cells formed a monolayer on the blend membrane, mild signs of inflammation were observed *in vivo*.[199]

Silk fibroin

Silk fibroin has been extensively used in regenerative medicine because of its optical properties, non-immunogenic response, flexible degradation standards and mechanical characteristics.[159,200] Silk fibroin membranes have been shown to support the formation of a confluent hCEC monolayer.[86,200] These hCEC-membrane constructs, with cells showing normal morphology and expression of characteristic markers, were trialed in a rabbit model.[200] Corneal transparency was maintained up to 6 weeks after transplantation without inflammatory reactions and the silk fibroin films remained optically transparent.[200]

Thermoresponsive polymers

Thermoresponsive polymers, such as poly(N-isopropylacrylamide) (PNIPAAm) and copolymers based on it, offer the advantage of thermally modulating their hyrophobic and hydrophilic properties which facilitates the detachment of cell

sheets without enzymatic digestion.[159] The use of a thermoresponsive polymer was first described by Hsiue et al.[140] In a temperature-mediated process, hCEC were seeded and cultured on hydrophobic poly(N-isopropylacrylamide) (PNIPAA)m-grafted surfaces at 37˚C. Once the culture temperature was lowered to 20˚C, the resulting hCEC sheet detached from the now hydrophilic PNIPAAm-grafted surfaces and were implanted in rabbit eyes together with an adhesive gelatin hydrogel disc. After swelling and biodegradation of the gelatin hydrogel disc, the transplanted hCEC sheet was fully attached to the posterior stroma and the corneas were clear, with near normal corneal thickness within two weeks.[140] While thermoresponsive polymers may offer interesting properties as a potential carrier, more detailed analysis on how the temperature change effects hCEC functionality is still lacking.

Other synthetic carriers

Recently, biodegradable electrospun poly(glycerol sebacate)-poly(ε-caprolactone) blend nanofibrous,[201] and poly(lactic-co-glycolic acid) scaffolds[202] were tested *in vitro* as possible carriers for hCEC by showing a monolayer of cultured hCEC with normal morphology. In addition, a hybrid carrier was constructed with a surface-initiated assembly technique: this hybrid was composed of basement membrane proteins (collagen IV and laminin), supported by a collagen-1 gel to mimic DM and a layer of stroma with a consistent thickness of 10μm, therefore similar in composition to a DSEK-graft but more similar to a DMEK-graft in terms of thickness.[203] Human CEC seeded on this carrier formed a monolayer and expressed continuous ZO-1 at their borders, showing that this technique could generate a biocompatible membrane with biomechanical properties similar to those of a native basement membrane and therefore suitable as a bioengineered corneal endothelial graft.

CELL INJECTION THERAPY

First approaches to cell injection therapy

Cultured CEC can also be directly injected into the anterior chamber of the eye, avoiding the need for a carrier entirely. This concept was first described by Mimura et al. using iron-endocytosed rabbit CEC, and later using spheres of CEC precursors.[204-206] Injection of hCEC precursors in a rabbit model confirmed the potential of this approach, as long as a postoperative prone position could be maintained.[207] To improve the number of cells attaching, Patel et al. investigated the incorporation of supermagnetic microspheres into cultured hCEC before injection and applied a magnetic field to direct cells towards the posterior cornea in an *ex vivo* model.[208]

In 2012, the use of ROCK inhibitor as an adjuvant to promote hCEC adhesion to the posterior cornea after cell injection was suggested,[209,210] after the same group reported that the ROCK inhibitor Y-27632 enhanced adhesion and inhibited apoptosis of monkey CEC *in vitro,* and enhanced corneal endothelial wound healing *in vivo* in animal models.[211,212]

In vivo application of cell injection in animal models

Additional work was conducted with monkey and rabbit models, to further explore the concept of cell injection therapy.[44,213,214] In the initial experiments, the recipient's cells were removed by scraping but Okumura et al. later showed, in a rabbit model, that a small descemetorhexis of 4 mm could be performed.[214] Recently, hCEC injection was compared with the hCEC delivered on a tissue-engineered graft of human origin in a rabbit model of bullous keratopathy.[215] Results showed that both approaches were effective in reversing corneal blindness in the rabbit model. Transplanted hCEC retained their hexagonal shape and expressed phenotypical human cell markers, regardless the modality of delivery. In another rabbit model, the injection of a CEC suspension was compared with the injection of CEC mini-sheets, which were aggregates of 4-10 CEC derived from the enzymatic dissociation of confluent cultured rabbit CEC with accutase.[216] CEC mini-sheet injection restored corneal clarity and thickness in the rabbit eye after 7 days of injection, while for single CEC suspension this was observed after 14 days of injection.

A combination of the "modified" dual media approach[47] and cell injection was recently explored by Ong et al. After storage of peeled DM-EC tissue in a growth-factor-depleted medium for 48 hours, non-cultured single hCEC obtained after enzymatic treatment of DM-EC showed a smaller cell morphology and an overall cellular yield compared to single cells derived from digestion of DM-EC stored in a F99-based medium with growth factors. After non-cultured single cells injection in a rabbit bullous keratopathy model, corneal thickness became increasingly thinner and corneas remained clear at 3 weeks after injection.[217] Unlike published methodologies, this approach does not require complex cellular propagation techniques.

Clinical trials

A milestone for endothelial cell injection was the first-in-man clinical trial conducted by the Kinoshita group. The first patient reported showed resolution in the corneal edema, and experienced an improvement in visual acuity from 0.04 to 1.0 Decimal Snellen best corrected visual acuity (BCVA).[218] Results of a group of 11 patients, that were treated for BK (FECD n=7), showed clinical improvement and an increase in ECD after injection of a ROCK inhibitor-supplemented cell suspension.[23] Following the mechanical removal of the aberrant ECM on the patient's DM and/or of the degenerated CEC in an area of 8-mm diameter, a suspension of 10^6 cells was injected into the patient's anterior chamber. After the procedure, patients were placed in a prone position for 3 hours, to allow the sedimentation of the injected hCEC onto the posterior surface of the cornea. At 24 weeks after cell injection, improvements in ECD, corneal thickness decrease, and best corrected visual acuity were observed. Two years after cell injection, the corneal thickness was less than 600 μm in 10 eyes out of 11, while each of the 11 eyes retained corneal transparency. No immune response to hCEC was observed.[23]

Within the limits of the small study group, the procedure was safe and no severe adverse events were reported. However, safety issues still need to be better defined before applying this technique on a large scale.[23,219] The fate of unattached cells and their effect upon entering the trabecular meshwork or the systemic circulation is not yet fully understood.

An alternative approach for cell injection therapy was recently presented by Parikumar et al. with a small case series of three patients.[220] They first placed a nanocomposite gel sheet in the anterior chamber[221] and then injected a suspension of hCEC precursors in the space between the posterior cornea and the gel sheet. The gel sheet was intended to facilitate cell attachment and was removed three days after cell injection. After 6 months, bullae in all three analyzed eyes had resolved and did not re-occur within the 18-month follow-up period. No information on ECD was presented in the study.

REGULATORY COMPLIANCE

Clinical application of cultured hCEC requires compliance with strict regulatory guidelines. These guidelines may not be identical in all countries as they are set by local regulatory authorities,[222-228] but have the common goal to ensure safety and reproducibility. Cell isolation and culture protocols must adhere to GMP requirements because these treatments are considered advanced therapeutic medicinal products (ATMP) according to the European legislation, human cells, tissues and cellular and tissue-based products (HCT/P) as for United States' FDA regulation, and regenerative medical products (RMP) for the Japanese guidelines. A major challenge for GMP compliance is the use of animal-free components to reduce the risk of disease transmission and the batch-to-batch variability.

Collagenases used for cell isolation are produced by different bacteria such as *Clostridium histolyticum*, but the production is related to the fermentation of animal-derived nutrients.[229] GMP-compliant enzymes used in the hCEC isolation process include Liberase TH, an enzymatic blend without animal-derived components and mainly composed by defined ratios of highly purified Collagenase I and Collagenase II and with a high percentage of Thermolysin.[46,49,215] A comparative analysis of Liberase TH with the commonly used research-grade Collagenase I showed no differences in terms of both optimal working concentration for the digestion of the DM-EC complex and overall cell concentration obtained per cornea.[46] Collagenase NB may be another GMP-compliant option, as this enzymatic blend of Collagenase I/Collagenase II with the addition of Clostripain[230] has been used for the isolation of cells from many tissues.[231-235] For DM-EC digestion, the use of TrypLE™ Select has been suggested for GMP-compliant protocols and authors described a similar dissociation rate of confluent hCEC between TrypLE™ Select and another TrypLE™ reagent.[46]

In the vast majority of reported hCEC culture protocols, culture medium is supplemented with animal-derived fetal bovine serum (FBS).[38,40,41,43,45-48,61,137] Use of Equafetal was approved for clinical trials by FDA and pharmaceutical regulatory agencies of UK and Japan. However, while it is derived from animals held in controlled diet and living conditions, it is still a source of animal

components.[46] Other studies reported culture medium supplementation with human-derived serum[236] and human platelet lysate,[237] or even the establishment of hCEC cultures expanded using serum-free culture media,[32,46] though the success rates were variable.

The materials used for coating of cell culture surfaces are another potential source of xeno-contamination. Recombinant proteins such as laminin-511 and laminin-521 are already available on the market,[90] while the natural-derived pericellular matrix from human decidua-derived mesenchymal cells (PCM-DM) has also been shown as a potential xeno-free substrate.[137]

Regulatory authorities require that a quality assessment must be performed on cultured hCEC used for clinical application, to guarantee safety and efficacy for the therapy used on humans. A clinical trial in human patients by Kinoshita et al. included quality assessment features for cultured hCEC used for cell injection, such as: cell viability, visual inspection, cell purity, functional assurance, confirmation of no contamination of the culture medium, and negative testing for bacteria, fungi and viruses.[23] Moreover, several cell surface antigens (CD98, CD166, CD340), as well as cell membrane proteins (SLC4A11), microRNAs and exosomes, have been identified and proposed for quality assessment of cultured hCEC for clinical applications.[238-243]

FUTURE PERSPECTIVES

Corneal transplantation will most likely remain the standard of care for endothelial diseases in the next decade. Motivated by the global shortage of corneal grafts, however, novel therapeutic treatment options have been explored over the last years to either use donor tissue more efficiently or to become independent of donor tissue at all.

For novel approaches based on cultured hCEC, as described in this review, larger clinical trials will be needed to answer questions regarding indication and safety. Potential clinical uptake of this approach will, however, more likely be hampered by costs, as ATMPs are typically very expensive. These regulations also apply to the carrier-based approach, which is currently still in the preclinical stage. Approval for a first-in-man clinical trial for tissue-engineered corneal grafts was recently granted by Health Sciences Authority in Singapore (Clinical Trial Certificate: CTC1800013) for the treatment of FECD and bullous keratopathy.[212]

To further pursue the transplantation of cultured hCEC on carriers, additional research efforts will need to be invested in developing suitable carriers. While natural carriers closely mimic the native DM, availability is limited and subjected to donor-to-donor variability. The focus may therefore be best placed on bioengineered carriers which would be a potentially unlimited source for tissue-engineered grafts, and with the possibility to finely tune the biomechanical properties. Current challenges for bioengineered grafts are ensuring sufficient cell adherence and pumping function of transplanted cells on the carriers.

An interesting future aspect of the cell-based approaches may be the possibility to apply non-HLA matched allogeneic cells.[244-246] The alloimmunogenicity of murine primary CEC (mpCEC) that were injected into the anterior chamber of a mouse CEC transplantation model, created by excising mpCEC from the recipient murine cornea.[247] Results showed that injected mpCEC did not provoke an allogeneic delayed-type hypersensitive (DTH) response. Moreover, mpCEC that adhered to the recipient murine cornea induced allograft tolerance 8 weeks after injection, indicating that mpCEC transplantation may have a degree of protection from allogeneic rejection.

In parallel to developing cell-based approaches, also other approaches will continue in pursuit of becoming more independent from donor tissue. *In vitro* results for in-office approaches to perform laser-based descemetorhexis or selective guttae removal have recently been presented,[248,249] and may be combined with topical medication of ROCK inhibitors.[44,207,218] For gene therapy for FECD different approaches have been investigated.[250,251] Until the above-mentioned approaches can be applied clinically, current tissue-efficient or tissue-sparing techniques such as Quarter-DMEK and Descemet stripping only will remain the main treatment alternatives to standard corneal transplantation.

CONCLUSION

In conclusion, promising new developments targeting endothelial donor tissue shortage are emerging. While the first clinical results for surgical-based approaches and cell injection therapy are available, engineered endothelial cell sheet transplantation is still in the pre-clinical stage. For all approaches, more studies are needed to evaluate whether these techniques have indeed the potential to complement the available corneal transplantation techniques as a treatment option for endothelial diseases.

REFERENCES

1. Joyce NC, Meklir B, Joyce SJ, Zieske JD. Cell cycle protein expression and proliferative status in human corneal cells. Invest Ophthalmol Vis Sci. 1996;37:645-655

2. Joyce NC, Harris DL, Mello DM. Mechanisms of mitotic inhibition in corneal endothelium: contact inhibition and TGF-beta2. Invest Ophthalmol Vis Sci. 2002;43:2152-2159

3. Joyce NC. Proliferative capacity of the corneal endothelium. Prog Retin Eye Res. 2003;22:359-389. doi:10.1016/s1350-9462(02)00065-4

4. Murphy C, Alvarado J, Juster R, Maglio M. Prenatal and postnatal cellularity of the human corneal endothelium. A quantitative histologic study. Invest Ophthalmol Vis Sci. 1984;25:312-322.

5. Bourne WM, Nelson LR, Hodge DO. Central corneal endothelial cell changes over a ten-year period. Invest Ophthalmol Vis Sci. 1997;38:779-782.

6. Gambato C, Longhin E, Catania AG, Lazzarini D, Parrozzani R, Midena E. Aging and corneal layers: an in vivo corneal confocal microscopy study. Graefes Arch Clin Exp Ophthalmol. 2015;253:267-275. doi:10.1007/s00417-014-2812-2

7. DelMonte DW, Kim T. Anatomy and physiology of the cornea. J Cataract Refract Surg 2011;37:588-598. doi:10.1016/j.jcrs.2010.12.037

8. Engelmann K, Bednarz J, Valtink M. Prospects for endothelial transplantation. Exp Eye Res. 2004;78:573-578. doi:10.1016/s0014-4835(03)00209-4

9. Schmedt T, Silva MM, Ziaei A, Jurkunas U. Molecular bases of corneal endothelial dystrophies. Exp Eye Res. 2012;95:24-34. doi:10.1016/j.exer.2011.08.002

10. Duman F, Kosker M, Suri K, Reddy JC, Ma JF, Hammersmith KM, Nagra PK, Rapuano CJ. Indications and outcomes of corneal transplantation in geriatric patients. Am J Ophthalmol. 2013;156:600-607 e602. doi:10.1016/j.ajo.2013.04.034

11. Melles GR, Ong TS, Ververs B, van der Wees J. Descemet membrane endothelial keratoplasty (DMEK). Cornea. 2006;25:987-990.doi:10.1097/01.ico.0000248385.16896.34

12. Melles GR, Dapena I. How to get started with standardized 'no-touch' Descemet membrane endothelial keratoplasty (DMEK). 1st ed. Netherlands Institute for Innovative Ocular Surgery, 2014.

13. Gain P, Jullienne R, He Z, Aldossary M, Acquart S, Cognasse F, Thuret G. Global Survey of Corneal Transplantation and Eye Banking. JAMA Ophthalmol. 2016;134:167-173. doi:10.1001/jamaophthalmol.2015.4776

14. Arbelaez JG, Price MO, Price FW, Jr. Long-term follow-up and complications of stripping descemet membrane without placement of graft in eyes with Fuchs endothelial dystrophy. Cornea. 2014;33:1295-1299. doi:10.1097/ICO.0000000000000270

15. Borkar DS, Veldman P, Colby KA. Treatment of Fuchs Endothelial Dystrophy by Descemet Stripping Without Endothelial Keratoplasty. Cornea. 2016;35:1267-1273. doi:10.1097/ICO.0000000000000915

16. Moloney G, Petsoglou C, Ball M, Kerdraon Y, Hollhumer R, Spiteri N, Beheregaray S, Hampson J, D'Souza M, Devasahayam RN. Descemetorhexis Without Grafting for Fuchs Endothelial Dystrophy-Supplementation With Topical Ripasudil. Cornea. 2017;36:642-648. doi:10.1097/ICO.0000000000001209

17. Muller TM, Lavy I, Baydoun L, Lie JT, Dapena I, Melles GR. Case Report of Quarter-Descemet Membrane Endothelial Keratoplasty for Fuchs Endothelial Dystrophy. Cornea. 2017;36:104-107. doi:10.1097/ICO.0000000000001008

18. Baydoun L, Zygoura V, Hsien S, Birbal RS, Spinozzi D, Lie JT, Ham L, Oellerich S, Melles GRJ. Clinical feasibility of using multiple grafts from a single donor for Quarter-DMEK. Acta Ophthalmol. 2018;96:e656-e658. doi:10.1111/aos.13720

19. Zygoura V, Baydoun L, Ham L, Bourgonje VJA, van Dijk K, Lie JT, Dapena I, Oellerich S, Melles GRJ. Quarter-Descemet membrane endothelial keratoplasty (Quarter-DMEK) for Fuchs endothelial corneal dystrophy: 6 months clinical outcome. Br J Ophthalmol 2018;102:1425-1430. doi:10.1136/bjophthalmol-2017-311398

20. Birbal RS, Ni Dhubhghaill S, Baydoun L, Ham L, Bourgonje VJA, Dapena I, Oellerich S, Melles GRJ. Quarter-Descemet Membrane Endothelial Keratoplasty: One- to Two-Year Clinical Outcomes. Cornea. 2020;39:277-282. doi:10.1097/ICO.0000000000002127

21. Peh GS, Beuerman RW, Colman A, Tan DT, Mehta JS. Human corneal endothelial cell expansion for corneal endothelium transplantation: an overview. Transplantation. 2011;91:811-819. doi:10.1097/TP.0b013e3182111f01

22. Okumura N, Kinoshita S, Koizumi N. Cell-based approach for treatment of corneal endothelial dysfunction. Cornea. 2014;33 Suppl 11:S37-41. doi:10.1097/ICO.0000000000000229

23. Kinoshita S, Koizumi N, Ueno M, Okumura N, Imai K, Tanaka H, Yamamoto Y, Nakamura T, Inatomi T, Bush J, Toda M, Hagiya M, Yokota I, Teramukai S, Sotozono C, Hamuro J. Injection of Cultured Cells with a ROCK Inhibitor for Bullous Keratopathy. N Engl J Med. 2018;378:995-1003. doi:10.1056/NEJMoa1712770

24. Mannagh J, Irving AR, Jr. Human corneal endothelium: growth in tissue cultures. Arch Ophthalmol. 1965;74:847-849. doi:10.1001/archopht.1965.00970040849023

25. Hyldahl L. Primary cell cultures from human embryonic corneas. J Cell Sci. 1984;66:343-351.

26. Engelmann K, Bohnke M, Friedl P. Isolation and long-term cultivation of human corneal endothelial cells. Invest Ophthalmol Vis Sci. 1988;29:1656-1662.

27. Pistsov MY, Sadovnikova E, Danilov SM. Human corneal endothelial cells: isolation, characterization and long-term cultivation. Exp Eye Res. 1988;47:403-414. doi:10.1016/0014-4835(88)90051-6

28. Yue BY, Sugar J, Gilboy JE, Elvart JL. Growth of human corneal endothelial cells in culture. Invest Ophthalmol Vis Sci. 1989;30:248-253.

29. Blake DA, Yu H, Young DL, Caldwell DR. Matrix stimulates the proliferation of human corneal endothelial cells in culture. Invest Ophthalmol Vis Sci. 1997;38:1119-1129.

30. Bednarz J, Rodokanaki-von Schrenck A, Engelmann K. Different characteristics of endothelial cells from central and peripheral human cornea in primary culture and after subculture. In Vitro Cell Dev Biol Anim. 1988;34:149-153. doi:10.1007/s11626-998-0097-7

31. Orwin EJ, Hubel A. In vitro culture characteristics of corneal epithelial, endothelial, and keratocyte cells in a native collagen matrix. Tissue Eng. 2000;6:307-319. doi:10.1089/107632700418038

32. Bednarz J, Doubilei V, Wollnik PC, Engelmann K. Effect of three different media on serum free culture of donor corneas and isolated human corneal endothelial cells. Br J Ophthalmol. 2001;85:1416-1420. doi:10.1136/bjo.85.12.1416

33. Miyata K, Drake J, Osakabe Y, Hosokawa Y, Hwang D, Soya K, Oshika T, Amano S. Effect of donor age on morphologic variation of cultured human corneal endothelial cells. Cornea. 2001;20:59-63. doi:10.1097/00003226-200101000-00012

34. Zhu C, Joyce NC. (2004) Proliferative response of corneal endothelial cells from young and older donors. Invest Ophthalmol Vis Sci 45:1743-1751. doi:10.1167/iovs.03-0814

35. Li W, Sabater AL, Chen YT, Hayashida Y, Chen SY, He H, Tseng SC. A novel method of isolation, preservation, and expansion of human corneal endothelial cells. Invest Ophthalmol Vis Sci. 2007;48:614-620. doi:10.1167/iovs.06-1126

36. Engler C, Kelliher C, Speck CL, Jun AS. Assessment of attachment factors for primary cultured human corneal endothelial cells. Cornea. 2009;28:1050-1054. doi:10.1097/ICO.0b013e3181a165a3

37. Mimura T, Yamagami S, Yokoo S, Usui T, Amano S. Selective isolation of young cells from human corneal endothelium by the sphere-forming assay. Tissue Eng Part C Methods. 2010;16:803-812. doi:10.1089/ten.TEC.2009.0608

38. Peh GS, Toh KP, Wu FY, Tan DT, Mehta JS. Cultivation of human corneal endothelial cells isolated from paired donor corneas. PLoS One. 2011;6:e28310. doi:10.1371/journal.pone.0028310

39. Nakahara M, Okumura N, Kay EP, Hagiya M, Imagawa K, Hosoda Y, Kinoshita S, Koizumi N. Corneal endothelial expansion promoted by human bone marrow mesenchymal stem cell-derived conditioned medium. PLoS One. 2013;8:e69009. doi:10.1371/journal.pone.0069009

40. Okumura N, Kay EP, Nakahara M, Hamuro J, Kinoshita S, Koizumi N. Inhibition of TGF-beta signaling enables human corneal endothelial cell expansion in vitro for use in regenerative medicine. PLoS One. 2013;8:e58000. doi:10.1371/journal.pone. 0058000

41. Peh GS, Toh KP, Ang HP, Seah XY, George BL, Mehta JS. Optimization of human corneal endothelial cell culture: density dependency of successful cultures in vitro. BMC Res Notes. 2013;6:176. doi:10.1186/1756-0500-6-176

42. Choi JS, Kim EY, Kim MJ, Khan FA, Giegengack M, D'Agostino R, Jr., Criswell T, Khang G, Soker S. Factors affecting successful isolation of human corneal endothelial cells for clinical use. Cell Transplant. 2014;23:845-854. doi:10.3727/ 096368913X664559

43. Peh GS, Chng Z, Ang HP, Cheng TY, Adnan K, Seah XY, George BL, Toh KP, Tan DT, Yam GH, Colman A, Mehta JS. Propagation of human corneal endothelial cells: a novel dual media approach. Cell Transplant. 2015;24:287-304. doi:10.3727/096368913X675719

44. Okumura N, Sakamoto Y, Fujii K, Kitano J, Nakano S, Tsujimoto Y, Nakamura S, Ueno M, Hagiya M, Hamuro J, Matsuyama A, Suzuki S, Shiina T, Kinoshita S, Koizumi N. Rho kinase inhibitor enables cell-based therapy for corneal endothelial dysfunction. Sci Rep. 2016;6:26113. doi:10.1038/srep26113

45. Parekh M, Ahmad S, Ruzza A, Ferrari S. Human Corneal Endothelial Cell Cultivation From Old Donor Corneas With Forced Attachment. Sci Rep. 2017;7:142. doi:10.1038/s41598-017-00209-5

46. Peh GSL, Ang HP, Lwin CN, Adnan K, George BL, Seah XY, Lin SJ, Bhogal M, Liu YC, Tan DT, Mehta JS. Regulatory Compliant Tissue-Engineered Human Corneal Endothelial Grafts Restore Corneal Function of Rabbits with Bullous Keratopathy. Sci Rep. 2017;7:14149. doi:10.1038/s41598-017-14723-z

47. Spinozzi D, Miron A, Bruinsma M, Lie JT, Dapena I, Oellerich S, Melles GRJ. Improving the success rate of human corneal endothelial cell cultures from single donor corneas with stabilization medium. Cell Tissue Bank. 2018;19:9-17. doi:10.1007/s10561-017-9665-y

48. Arnalich-Montiel F, Moratilla A, Fuentes-Julian S, Aparicio V, Cadenas Martin M, Peh G, Mehta JS, Adnan K, Porrua L, Perez-Sarriegui A, De Miguel MP. Treatment of corneal endothelial damage in a rabbit model with a bioengineered graft using human decellularized corneal lamina and cultured human corneal endothelium. PLoS One. 2019;14:e0225480. doi:10.1371/journal.pone.0225480

49. Wahlig S, Peh GSL, Adnan K, Ang HP, Lwin CN, Morales-Wong F, Ong HS, Lovatt M, Mehta JS. Optimisation of Storage and Transportation Conditions of Cultured Corneal Endothelial Cells for Cell Replacement Therapy. Sci Rep. 2020;10:1681. doi:10.1038/s41598-020-58700-5

50. Roy O, Leclerc VB, Bourget JM, Theriault M, Proulx S. Understanding the process of corneal endothelial morphological change in vitro. Invest Ophthalmol Vis Sci. 2015;56:1228-1237. doi:10.1167/iovs.14-16166

51. Engelmann K, Friedl P. Optimization of culture conditions for human corneal endothelial cells. In Vitro Cell Dev Biol. 1989;25:1065-1072. doi:10.1007/BF02624143

52. Navaratnam J, Utheim TP, Rajasekhar VK, Shahdadfar A. Substrates for Expansion of Corneal Endothelial Cells towards Bioengineering of Human Corneal Endothelium. J Funct Biomater. 2015;6:917-945. doi:10.3390/jfb6030917

53. Van den Bogerd B, Dhubhghaill SN, Koppen C, Tassignon MJ, Zakaria N. A review of the evidence for in vivo corneal endothelial regeneration. Surv Ophthalmol. 2018;63:149-165. doi:10.1016/j.survophthal.2017.07.004

54. Joyce NC, Zhu CC. Human corneal endothelial cell proliferation: potential for use in regenerative medicine. Cornea. 2004;23:S8-S19.doi:10.1097/01.ico.0000136666. 63870.18

55. Konomi K, Zhu C, Harris D, Joyce NC. Comparison of the proliferative capacity of human corneal endothelial cells from the central and peripheral areas. Invest Ophthalmol Vis Sci. 2005;46:4086-4091. doi:10.1167/iovs.05-0245

56. Enomoto K, Mimura T, Harris DL, Joyce NC. Age differences in cyclin-dependent kinase inhibitor expression and rb hyperphosphorylation in human corneal endothelial cells. Invest Ophthalmol Vis Sci. 2006;47:4330-4340. doi:10.1167/iovs.05-1581

57. Konomi K, Joyce NC. Age and topographical comparison of telomere lengths in human corneal endothelial cells. Mol Vis. 2007;13:1251-1258.

58. Song Z, Wang Y, Xie L, Zang X, Yin H. Expression of senescence-related genes in human corneal endothelial cells. Mol Vis. 2008;14:161-170.

59. Tripathi RC, Tripathi BJ. Human trabecular endothelium, corneal endothelium, keratocytes, and scleral fibroblasts in primary cell culture. A comparative study of growth characteristics, morphology, and phagocytic activity by light and scanning electron microscopy. Exp Eye Res. 1982;35:611-624. doi:10.1016/s0014-4835(82)80074-2

60. Chen KH, Azar D, Joyce NC. Transplantation of adult human corneal endothelium ex vivo: a morphologic study. Cornea. 2001;20:731-737. doi:10.1097/00003226-200110000-00012

61. Parekh M, Peh G, Mehta JS, Ramos T, Ponzin D, Ahmad S, Ferrari S. Passaging capability of human corneal endothelial cells derived from old donors with and without accelerating cell attachment. Exp Eye Res. 2019;189:107814. doi:10.1016/j.exer.2019.107814

62. Mimura T, Joyce NC. Replication competence and senescence in central and peripheral human corneal endothelium. Invest Ophthalmol Vis Sci. 2006;47:1387-1396. doi:10.1167/iovs.05-1199

63. Gao Y, Zhou Q, Qu M, Yang L, Wang Y, Shi W. In vitro culture of human fetal corneal endothelial cells. Graefes Arch Clin Exp Ophthalmol. 2011;249:663-669. doi:10.1007/s00417-010-1547-y

64. Aboalchamat B, Engelmann K, Bohnke M, Eggli P, Bednarz J. Morphological and functional analysis of immortalized human corneal endothelial cells after transplantation. Exp Eye Res. 1999;69:547-553. doi:10.1006/exer.1999.0736

65. Bednarz J, Teifel M, Friedl P, Engelmann K. Immortalization of human corneal endothelial cells using electroporation protocol optimized for human corneal endothelial and human retinal pigment epithelial cells. Acta Ophthalmol Scand. 2000;78:130-136. doi:10.1034/j.1600-0420.2000.078002130.x

66. Joyce NC, Harris DL. Decreasing expression of the G1-phase inhibitors, p21Cip1 and p16INK4a, promotes division of corneal endothelial cells from older donors. Mol Vis. 2010;16:897-906.

67. Yokoi T, Seko Y, Yokoi T, Makino H, Hatou S, Yamada M, Kiyono T, Umezawa A, Nishina H, Azuma N. Establishment of functioning human corneal endothelial cell line with high growth potential. PLoS One. 2012;7:e29677. doi:10.1371/journal.pone.0029677

68. Fan T, Ma X, Zhao J, Wen Q, Hu X, Yu H, Shi W. Transplantation of tissue-engineered human corneal endothelium in cat models. Mol Vis. 2013;19:400-407.

69. Shao C, Fu Y, Lu W, Fan X. Bone marrow-derived endothelial progenitor cells: a promising therapeutic alternative for corneal endothelial dysfunction. Cells Tissues Organs. 2011;193:253-263. doi:10.1159/000319797

70. Joyce NC, Harris DL, Markov V, Zhang Z, Saitta B. Potential of human umbilical cord blood mesenchymal stem cells to heal damaged corneal endothelium. Mol Vis. 2012;18:547-564.

71. Dai Y, Guo Y, Wang C, Liu Q, Yang Y, Li S, Guo X, Lian R, Yu R, Liu H, Chen J. Non-genetic direct reprogramming and biomimetic platforms in a preliminary study for adipose-derived stem cells into corneal endothelia-like cells. PLoS One. 2014;9:e109856. doi:10.1371/journal.pone.0109856

72. Zhang K, Pang K, Wu X. Isolation and transplantation of corneal endothelial cell-like cells derived from in-vitro-differentiated human embryonic stem cells. Stem Cells Dev. 2014;23:1340-1354. doi:10.1089/scd.2013.0510

73. Chen P, Chen JZ, Shao CY, Li CY, Zhang YD, Lu WJ, Fu Y, Gu P, Fan X. Treatment with retinoic acid and lens epithelial cell-conditioned medium in vitro directed the differentiation of pluripotent stem cells towards corneal endothelial cell-like cells. Exp Ther Med. 2015;9:351-360. doi:10.3892/etm.2014.2103

74. Yokoo S, Yamagami S, Yanagi Y, Uchida S, Mimura T, Usui T, Amano S. Human corneal endothelial cell precursors isolated by sphere-forming assay. Invest Ophthalmol Vis Sci. 2005;46:1626-1631. doi:10.1167/iovs.04-1263

75. Yam GH, Seah X, Yusoff N, Setiawan M, Wahlig S, Htoon HM, Peh GSL, Kocaba V, Mehta JS. Characterization of Human Transition Zone Reveals a Putative Progenitor-Enriched Niche of Corneal Endothelium. Cells 2019;8. doi:10.3390/cells8101244

76. Whikehart DR, Parikh CH, Vaughn AV, Mishler K, Edelhauser HF. Evidence suggesting the existence of stem cells for the human corneal endothelium. Mol Vis. 2005;11:816-824.

77. Mimura T, Yamagami S, Amano S. Corneal endothelial regeneration and tissue engineering. Prog Retin Eye Res. 2013;35:1-17. doi:10.1016/j.preteyeres.2013.01.003

78. Yu WY, Sheridan C, Grierson I, Mason S, Kearns V, Lo AC, Wong D. Progenitors for the corneal endothelium and trabecular meshwork: a potential source for personalized stem cell therapy in corneal endothelial diseases and glaucoma. J Biomed Biotechnol. 2011;2011:412743. doi:10.1155/2011/412743

79. He Z, Campolmi N, Gain P, Ha Thi BM, Dumollard JM, Duband S, Peoc'h M, Piselli S, Garraud O, Thuret G. Revisited microanatomy of the corneal endothelial periphery:

new evidence for continuous centripetal migration of endothelial cells in humans. Stem Cells. 2012;30:2523-2534. doi:10.1002/stem.1212

80. Yoon JJ, Wang EF, Ismail S, McGhee JJ, Sherwin T. Sphere-forming cells from peripheral cornea demonstrate polarity and directed cell migration. Cell Biol Int. 2013;37:949-960. doi:10.1002/cbin.10119

81. Noh JW, Kim JJ, Hyon JY, Chung ES, Chung TY, Yi K, Wee WR, Shin YJ. Stemness characteristics of human corneal endothelial cells cultured in various media. Eye Contact Lens. 2015;41:190-196. doi:10.1097/ICL.0000000000000110

82. Zavala J, Lopez Jaime GR, Rodriguez Barrientos CA, Valdez-Garcia J. Corneal endothelium: developmental strategies for regeneration. Eye (Lond). 2013;27:579-588. doi:10.1038/eye.2013.15

83. Ishino Y, Sano Y, Nakamura T, Connon CJ, Rigby H, Fullwood NJ, Kinoshita S. Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. Invest Ophthalmol Vis Sci. 2004;45:800-806. doi:10.1167/iovs.03-0016

84. Yoeruek E, Saygili O, Spitzer MS, Tatar O, Bartz-Schmidt KU, Szurman P. Human anterior lens capsule as carrier matrix for cultivated human corneal endothelial cells. Cornea. 2009;28:416-420. doi:10.1097/ICO.0b013e31818c2c36

85. Choi JS, Williams JK, Greven M, Walter KA, Laber PW, Khang G, Soker S. Bioengineering endothelialized neo-corneas using donor-derived corneal endothelial cells and decellularized corneal stroma. Biomaterials. 2010;31:6738-6745. doi:10.1016/j.biomaterials.2010.05.020

86. Madden PW, Lai JN, George KA, Giovenco T, Harkin DG, Chirila TV. Human corneal endothelial cell growth on a silk fibroin membrane. Biomaterials. 2011;32:4076-4084. doi:10.1016/j.biomaterials.2010.12.034

87. Shima N, Kimoto M, Yamaguchi M, Yamagami S. Increased proliferation and replicative lifespan of isolated human corneal endothelial cells with L-ascorbic acid 2-phosphate. Invest Ophthalmol Vis Sci. 2011;52:8711-8717. doi:10.1167/iovs.11-7592

88. Levis HJ, Peh GS, Toh KP, Poh R, Shortt AJ, Drake RA, Mehta JS, Daniels JT. Plastic compressed collagen as a novel carrier for expanded human corneal endothelial cells for transplantation. PLoS One. 2012;7:e50993. doi:10.1371/journal.pone.0050993

89. Kim E, Kim JJ, Hyon JY, Chung ES, Chung TY, Yi K, Wee WR, Shin YJ. The effects of different culture media on human corneal endothelial cells. Invest Ophthalmol Vis Sci. 2014;55:5099-5108. doi:10.1167/iovs.14-14564

90. Okumura N, Kakutani K, Numata R, Nakahara M, Schlotzer-Schrehardt U, Kruse F, Kinoshita S, Koizumi N. Laminin-511 and -521 enable efficient in vitro expansion of human corneal endothelial cells. Invest Ophthalmol Vis Sci. 2015;56:2933-2942. doi:10.1167/iovs.14-15163

91. Tsaousis KT, Kopsachilis N, Tsinopoulos IT, Dimitrakos SA, Kruse FE, Welge-Luessen U. In Vitro Study of the Deturgescence Ability of Cultivated Human Corneal Endothelial Cells. Cornea. 2016;35:669-672. doi:10.1097/ICO.0000000000000792

92. Yamaguchi M, Shima N, Kimoto M, Ebihara N, Murakami A, Yamagami S. Optimization of Cultured Human Corneal Endothelial Cell Sheet Transplantation and Post-Operative Sheet Evaluation in a Rabbit Model. Curr Eye Res. 2016;41:1178-1184. doi:10.3109/02713683.2015.1101774

93. Okumura N, Inoue R, Kakutani K, Nakahara M, Kinoshita S, Hamuro J, Koizumi N. Corneal Endothelial Cells Have an Absolute Requirement for Cysteine for Survival. Cornea. 2017;36:988-994. doi:10.1097/ICO.0000000000001242

94. Senoo T, Obara Y, Joyce NC. EDTA: a promoter of proliferation in human corneal endothelium. Invest Ophthalmol Vis Sci. 2000;41:2930-2935

95. Amano S. Transplantation of cultured human corneal endothelial cells. Cornea. 2003;22:S66-74. doi:10.1097/00003226-200310001-00010

96. Mimura T, Yamagami S, Yokoo S, Usui T, Tanaka K, Hattori S, Irie S, Miyata K, Araie M, Amano S. Cultured human corneal endothelial cell transplantation with a collagen sheet in a rabbit model. Invest Ophthalmol Vis Sci. 2004;45:2992-2997. doi:10.1167/iovs.03-1174

97. Hitani K, Yokoo S, Honda N, Usui T, Yamagami S, Amano S. Transplantation of a sheet of human corneal endothelial cell in a rabbit model. Mol Vis. 2008;14:1-9

98. Lu X, Chen D, Liu Z, Li C, Liu Y, Zhou J, Wan P, Mou YG, Wang Z. Enhanced survival in vitro of human corneal endothelial cells using mouse embryonic stem cell conditioned medium. Mol Vis. 2010;16:611-622. doi:10.1167/2.7.611

99. Yamaguchi M, Ebihara N, Shima N, Kimoto M, Funaki T, Yokoo S, Murakami A, Yamagami S. Adhesion, migration, and proliferation of cultured human corneal endothelial cells by laminin-5. Invest Ophthalmol Vis Sci. 2011;52:679-684. doi:10.1167/iovs.10-5555

100. Okumura N, Kusakabe A, Hirano H, Inoue R, Okazaki Y, Nakano S, Kinoshita S, Koizumi N. Density-gradient centrifugation enables the purification of cultured corneal endothelial cells for cell therapy by eliminating senescent cells. Sci Rep. 2015;5:15005. doi:10.1038/srep15005

101. Proulx S, Brunette I. Methods being developed for preparation, delivery and transplantation of a tissue-engineered corneal endothelium. Exp Eye Res. 2012;95:68-75. doi:10.1016/j.exer.2011.06.013

102. Bartakova A, Kuzmenko O, Alvarez-Delfin K, Kunzevitzky NJ, Goldberg JL. A Cell Culture Approach to Optimized Human Corneal Endothelial Cell Function. Invest Ophthalmol Vis Sci. 2018;59:1617-1629. doi:10.1167/iovs.17-23637

103. Feizi S, Soheili ZS, Bagheri A, Balagholi S, Mohammadian A, Rezaei-Kanavi M, Ahmadieh H, Samiei S, Negahban K. Effect of amniotic fluid on the in vitro culture of human corneal endothelial cells. Exp Eye Res. 2014;122:132-140. doi:10.1016/j.exer.2014.04.002

104. Zhu YT, Chen HC, Chen SY, Tseng SC. Nuclear p120 catenin unlocks mitotic block of contact-inhibited human corneal endothelial monolayers without disrupting adherent junctions. J Cell Sci. 2012;125:3636-3648. doi:10.1242/jcs.103267

105. Joko T, Shiraishi A, Akune Y, Tokumaru S, Kobayashi T, Miyata K, Ohashi Y. Involvement of P38MAPK in human corneal endothelial cell migration induced by TGF-beta(2). Exp Eye Res. 2013;108:23-32. doi:10.1016/j.exer.2012.11.018

106. Lee HT, Lee JG, Na M, Kay EP. FGF-2 induced by interleukin-1 beta through the action of phosphatidylinositol 3-kinase mediates endothelial mesenchymal transformation in corneal endothelial cells. J Biol Chem. 2004;279:32325-32332. doi:10.1074/jbc.M405208200

107. Lee JG, Kay EP. Common and distinct pathways for cellular activities in FGF-2 signaling induced by IL-1beta in corneal endothelial cells. Invest Ophthalmol Vis Sci. 2009;50:2067-2076. doi:10.1167/iovs.08-3135

108. Song JS, Lee JG, Kay EP. Induction of FGF-2 synthesis by IL-1beta in aqueous humor through P13-kinase and p38 in rabbit corneal endothelium. Invest Ophthalmol Vis Sci. 2010;51:822-829. doi:10.1167/iovs.09-4240

109. Lee JG, Ko MK, Kay EP. Endothelial mesenchymal transformation mediated by IL-1beta-induced FGF-2 in corneal endothelial cells. Exp Eye Res. 2012;95:35-39. doi:10.1016/j.exer.2011.08.003

110. Lee JG, Kay EP. NF-kappaB is the transcription factor for FGF-2 that causes endothelial mesenchymal transformation in cornea. Invest Ophthalmol Vis Sci. 2012;53:1530-1538. doi:10.1167/iovs.11-9102

111. Nakano Y, Oyamada M, Dai P, Nakagami T, Kinoshita S, Takamatsu T. Connexin43 knockdown accelerates wound healing but inhibits mesenchymal transition after corneal endothelial injury in vivo. Invest Ophthalmol Vis Sci. 2008;49:93-104. doi:10.1167/iovs.07-0255

112. Li C, Dong F, Jia Y, Du H, Dong N, Xu Y, Wang S, Wu H, Liu Z, Li W. Notch signal regulates corneal endothelial-to-mesenchymal transition. Am J Pathol. 2013;183:786-795. doi:10.1016/j.ajpath.2013.05.025

113. Nomura H, Ishiguro T, Morimoto S. Studies on L-ascorbic acid derivatives. 3. Bis(L-ascorbic acid-3,3')phosphate and L-ascorbic acid 2-phosphate. Chem Pharm Bull (Tokyo). 1969;17:387-393. doi:10.1248/cpb.17.387

114. Utoguchi N, Ikeda K, Saeki K, Oka N, Mizuguchi H, Kubo K, Nakagawa S, Mayumi T. Ascorbic acid stimulates barrier function of cultured endothelial cell monolayer. J Cell Physiol. 1995;163:393-399. doi:10.1002/jcp.1041630219

115. Hata R, Senoo H. L-ascorbic acid 2-phosphate stimulates collagen accumulation, cell proliferation, and formation of a three-dimensional tissuelike substance by skin fibroblasts. J Cell Physiol. 1989;138:8-16. doi:10.1002/jcp.1041380103

116. Takamizawa S, Maehata Y, Imai K, Senoo H, Sato S, Hata R. Effects of ascorbic acid and ascorbic acid 2-phosphate, a long-acting vitamin C derivative, on the proliferation and differentiation of human osteoblast-like cells. Cell Biol Int. 2004;28:255-265. doi:10.1016/j.cellbi.2004.01.010

117. Kimoto M, Shima N, Yamaguchi M, Amano S, Yamagami S. Role of hepatocyte growth factor in promoting the growth of human corneal endothelial cells stimulated by L-ascorbic acid 2-phosphate. Invest Ophthalmol Vis Sci. 2012;53:7583-7589. doi:10.1167/iovs.12-10146

118. Kefalides NA, Denduchis B. Structural components of epithelial and endothelial basement membranes. Biochemistry. 1969;8:4613-4621. doi:10.1021/bi00839a057

119. Carlson EC, Meezan E, Brendel K, Kenney MC. Ultrastructural analyses of control and enzyme-treated isolated renal basement membranes. Anat Rec. 1981;200:421-436. doi:10.1002/ar.1092000405

120. Sage H, Pritzl P, Bornstein P. Secretory phenotypes of endothelial cells in culture: comparison of aortic, venous, capillary, and corneal endothelium. Arteriosclerosis. 1981;1:427-442. doi:10.1161/01.atv.1.6.427

121. Labermeier U, Kenney MC. The presence of EC collagen and type IV collagen in bovine Descemet's membranes. Biochem Biophys Res Commun. 1983;116:619-625. doi:10.1016/0006-291x(83)90569-7

122. Benya PD, Padilla SR. Isolation and characterization of type VIII collagen synthesized by cultured rabbit corneal endothelial cells. A conventional structure replaces the interrupted-helix model. J Biol Chem. 1986;261:4160-4169.

123. Shuttleworth CA. Type VIII collagen. Int J Biochem Cell Biol. 1997;29:1145-1148. doi:10.1016/s1357-2725(97)00033-2

124. Samples JR, Binder PS, Nayak SK. Propagation of human corneal endothelium in vitro effect of growth factors. Exp Eye Res. 1991;52:121-128. doi:10.1016/0014-4835(91)90252-a

125. Underwood PA, Bennett FA. The effect of extracellular matrix molecules on the in vitro behavior of bovine endothelial cells. Exp Cell Res. 1993;205:311-319. doi:10.1006/excr.1993.1091

126. Rieck P, Oliver L, Engelmann K, Fuhrmann G, Hartmann C, Courtois Y. The role of exogenous/endogenous basic fibroblast growth factor (FGF2) and transforming growth factor beta (TGF beta-1) on human corneal endothelial cells proliferation in vitro. Exp Cell Res. 1995;220:36-46. doi:10.1006/excr.1995.1289

127. Akhta N, Carlso S, Pesarini A, Ambulos N, Passaniti A. Extracellular matrix-derived angiogenic factor(s) inhibit endothelial cell proliferation, enhance differentiation, and stimulate angiogenesis in vivo. Endothelium. 2001;8:221-234. doi:10.1080/10623320109051567

128. Ide T, Nishida K, Yamato M, Sumide T, Utsumi M, Nozaki T, Kikuchi A, Okano T, Tano Y. Structural characterization of bioengineered human corneal endothelial cell sheets fabricated on temperature-responsive culture dishes. Biomaterials. 2006;27:607-614. doi:10.1016/j.biomaterials.2005.06.005

129. Gotze T, Valtink M, Nitschke M, Gramm S, Hanke T, Engelmann K, Werner C. Cultivation of an immortalized human corneal endothelial cell population and two distinct clonal subpopulations on thermo-responsive carriers. Graefes Arch Clin Exp Ophthalmol. 2008;246:1575-1583. doi:10.1007/s00417-008-0904-6

130. Yoeruek E, Bayyoud T, Maurus C, Hofmann J, Spitzer MS, Bartz-Schmidt KU, Szurman P. Decellularization of porcine corneas and repopulation with human corneal cells for tissue-engineered xenografts. Acta Ophthalmol. 2012;90:e125-131. doi:10.1111/j.1755-3768.2011.02261.x

131. Choi JS, Kim EY, Kim MJ, Giegengack M, Khan FA, Khang G, Soker S. In vitro evaluation of the interactions between human corneal endothelial cells and extracellular matrix proteins. Biomed Mater. 2013;8:014108. doi:10.1088/1748-6041/8/1/014108

132. Palchesko RN, Lathrop KL, Funderburgh JL, Feinberg AW. In vitro expansion of corneal endothelial cells on biomimetic substrates. Sci Rep. 2015;5:7955. doi:10.1038/srep07955

133. Li S, Han Y, Lei H, Zeng Y, Cui Z, Zeng Q, Zhu D, Lian R, Zhang J, Chen Z, Chen J. In vitro biomimetic platforms featuring a perfusion system and 3D spheroid culture

promote the construction of tissue-engineered corneal endothelial layers. Sci Rep. 2017;7:777. doi:10.1038/s41598-017-00914-1

134. Insler MS, Lopez JG. Microcarrier cell culture of neonatal human corneal endothelium. Curr Eye Res. 1990;9:23-30. doi:10.3109/02713689009000051

135. Sumide T, Nishida K, Yamato M, Ide T, Hayashida Y, Watanabe K, Yang J, Kohno C, Kikuchi A, Maeda N, Watanabe H, Okano T, Tano Y. Functional human corneal endothelial cell sheets harvested from temperature-responsive culture surfaces. FASEB J. 2006;20:392-394. doi:10.1096/fj.04-3035fje

136. Kopsachilis N, Tsinopoulos I, Tourtas T, Kruse FE, Luessen UW. Descemet's membrane substrate from human donor lens anterior capsule. Clin Exp Ophthalmol. 2012;40:187-194. doi:10.1111/j.1442-9071.2011.02678.x

137. Numata R, Okumura N, Nakahara M, Ueno M, Kinoshita S, Kanematsu D, Kanemura Y, Sasai Y, Koizumi N. Cultivation of corneal endothelial cells on a pericellular matrix prepared from human decidua-derived mesenchymal cells. PLoS One. 2014;9:e88169. doi:10.1371/journal.pone.0088169

138. Yoshida J, Oshikata-Miyazaki A, Yokoo S, Yamagami S, Takezawa T, Amano S. Development and evaluation of porcine atelocollagen vitrigel membrane with a spherical curve and transplantable artificial corneal endothelial grafts. Invest Ophthalmol Vis Sci. 2014;55:4975-4981. doi:10.1167/iovs.14-14211

139. Nayak SK, Binder PS. The growth of endothelium from human corneal rims in tissue culture. Invest Ophthalmol Vis Sci. 1984;25:1213-1216.

140. Hsiue GH, Lai JY, Chen KH, Hsu WM. A novel strategy for corneal endothelial reconstruction with a bioengineered cell sheet. Transplantation. 2006;81:473-476. doi:10.1097/01.tp.0000194864.13539.2c

141. Lai JY, Lu PL, Chen KH, Tabata Y, Hsiue GH. Effect of charge and molecular weight on the functionality of gelatin carriers for corneal endothelial cell therapy. Biomacromolecules. 2006;7:1836-1844. doi:10.1021/bm0601575

142. Watanabe R, Hayashi R, Kimura Y, Tanaka Y, Kageyama T, Hara S, Tabata Y, Nishida K. A novel gelatin hydrogel carrier sheet for corneal endothelial transplantation. Tissue Eng Part A. 2011;17:2213-2219. doi:10.1089/ten.TEA.2010.0568

143. Amano S, Mimura T, Yamagami S, Osakabe Y, Miyata K. Properties of corneas reconstructed with cultured human corneal endothelial cells and human corneal stroma. Jpn J Ophthalmol. 2005;49:448-452. doi:10.1007/s10384-005-0245-5

144. Scheele S, Nystrom A, Durbeej M, Talts JF, Ekblom M, Ekblom P. Laminin isoforms in development and disease. J Mol Med (Berl). 2007;85:825-836. doi:10.1007/s00109-007-0182-5

145. Tzu J, Marinkovich MP. Bridging structure with function: structural, regulatory, and developmental role of laminins. Int J Biochem Cell Biol. 2008;40:199-214. doi:10.1016/j.biocel.2007.07.015

146. Miyazaki T, Futaki S, Suemori H, Taniguchi Y, Yamada M, Kawasaki M, Hayashi M, Kumagai H, Nakatsuji N, Sekiguchi K, Kawase E. Laminin E8 fragments support efficient adhesion and expansion of dissociated human pluripotent stem cells. Nat Commun. 2012;3:1236. doi:10.1038/ncomms2231

147. Nagase T, Ueno M, Matsumura M, Muguruma K, Ohgushi M, Kondo N, Kanematsu D, Kanemura Y, Sasai Y. Pericellular matrix of decidua-derived mesenchymal cells: a potent human-derived substrate for the maintenance culture of human ES cells. Dev Dyn. 2009;238:1118-1130. doi:10.1002/dvdy.21944

148. Kanematsu D, Shofuda T, Yamamoto A, Ban C, Ueda T, Yamasaki M, Kanemura Y. Isolation and cellular properties of mesenchymal cells derived from the decidua of human term placenta. Differentiation. 2011;82:77-88. doi:10.1016/j.diff.2011.05.010

149. Fukusumi H, Shofuda T, Kanematsu D, Yamamoto A, Suemizu H, Nakamura M, Yamasaki M, Ohgushi M, Sasai Y, Kanemura Y. Feeder-free generation and long-term culture of human induced pluripotent stem cells using pericellular matrix of decidua derived mesenchymal cells. PLoS One. 2013;8:e55226. doi:10.1371/journal.pone.0055226

150. Mimura T, Amano S, Usui T, Araie M, Ono K, Akihiro H, Yokoo S, Yamagami S. Transplantation of corneas reconstructed with cultured adult human corneal endothelial cells in nude rats. Exp Eye Res. 2004;79:231-237. doi:10.1016/j.exer.2004.05.001

151. Fu Y, Fan X, Chen P, Shao C, Lu W. Reconstruction of a tissue-engineered cornea with porcine corneal acellular matrix as the scaffold. Cells Tissues Organs. 2010;191:193-202. doi:10.1159/000235680

152. Bayyoud T, Thaler S, Hofmann J, Maurus C, Spitzer MS, Bartz-Schmidt KU, Szurman P, Yoeruek E. Decellularized bovine corneal posterior lamellae as carrier matrix for cultivated human corneal endothelial cells. Curr Eye Res. 2012;37:179-186. doi:10.3109/02713683.2011.644382

153. He Z, Forest F, Bernard A, Gauthier AS, Montard R, Peoc'h M, Jumelle C, Courrier E, Perrache C, Gain P, Thuret G. Cutting and Decellularization of Multiple Corneal

Stromal Lamellae for the Bioengineering of Endothelial Grafts. Invest Ophthalmol Vis Sci. 2016;57:6639-6651. doi:10.1167/iovs.16-20256

154. Lai JY, Chen KH, Hsiue GH. Tissue-engineered human corneal endothelial cell sheet transplantation in a rabbit model using functional biomaterials. Transplantation. 2007;84:1222-1232. doi:10.1097/01.tp.0000287336.09848.39

155. Mi S, Chen B, Wright B, Connon CJ. Plastic compression of a collagen gel forms a much improved scaffold for ocular surface tissue engineering over conventional collagen gels. J Biomed Mater Res A. 2010;95:447-453. doi:10.1002/jbm.a.32861

156. Niu G, Choi JS, Wang Z, Skardal A, Giegengack M, Soker S. Heparin-modified gelatin scaffolds for human corneal endothelial cell transplantation. Biomaterials. 2014;35:4005-4014. doi:10.1016/j.biomaterials.2014.01.033

157. Kong B, Sun W, Chen G, Tang S, Li M, Shao Z, Mi S. Tissue-engineered cornea constructed with compressed collagen and laser-perforated electrospun mat. Sci Rep. 2017;7:970. doi:10.1038/s41598-017-01072-0

158. Rizwan M, Peh GSL, Ang HP, Lwin NC, Adnan K, Mehta JS, Tan WS, Yim EKF. Sequentially-crosslinked bioactive hydrogels as nano-patterned substrates with customizable stiffness and degradation for corneal tissue engineering applications. Biomaterials. 2017;120:139-154. doi:10.1016/j.biomaterials.2016.12.026

159. Chen Z, You J, Liu X, Cooper S, Hodge C, Sutton G, Crook JM, Wallace GG. Biomaterials for corneal bioengineering. Biomed Mater. 2018;13:032002. doi:10.1088/1748-605X/aa92d2

160. Insler MS, Lopez JG. Transplantation of cultured human neonatal corneal endothelium. Curr Eye Res. 1986;5:967-972. doi:10.3109/02713688608995178

161. Insler MS, Lopez JG. Heterologous transplantation versus enhancement of human corneal endothelium. Cornea. 1991;10:136-148. doi:10.1097/00003226-199103000-00009

162. Insler MS, Lopez JG. Extended incubation times improve corneal endothelial cell transplantation success. Invest Ophthalmol Vis Sci. 1991;32:1828-1836.

163. Gospodarowicz D, Greenburg G. The coating of bovine and rabbit corneas denuded of their endothelium with bovine corneal endothelial cells. Exp Eye Res. 1979;28:249-265. doi:10.1016/0014-4835(79)90087-3

164. Engelmann K, Drexler D, Bohnke M. Transplantation of adult human or porcine corneal endothelial cells onto human recipients in vitro. Part I: Cell culturing and transplantation procedure. Cornea. 1999;18:199-206. doi:10.1097/00003226-199903000-00010

165. Proulx S, Bensaoula T, Nada O, Audet C, d'Arc Uwamaliya J, Devaux A, Allaire G, Germain L, Brunette I. Transplantation of a tissue-engineered corneal endothelium reconstructed on a devitalized carrier in the feline model. Invest Ophthalmol Vis Sci. 2009;50:2686-2694. doi:10.1167/iovs.08-2793

166. Spinozzi D, Miron A, Lie JT, Rafat M, Lagali N, Melles GRJ, Dhubhghaill SN, Dapena I, Oellerich S. In Vitro Evaluation and Transplantation of Human Corneal Endothelial Cells Cultured on Biocompatible Carriers. Cell Transplant. 2020;29:963689720923577. doi:10.1177/0963689720923577

167. Parmigiani C, McAvoy J. Localisation of laminin and fibronectin during rat lens morphogenesis. Differentiation. 1984;28:53-61. doi:10.1111/j.1432-0436.1984.tb00266.x

168. Cammarata PR, Cantu-Crouch D, Oakford L, Morrill A. Macromolecular organization of bovine lens capsule. Tissue Cell. 1986;18:83-97. doi:10.1016/0040-8166(86)90009-1

169. Kohno T, Sorgente N, Ishibashi T, Goodnight R, Ryan SJ. Immunofluorescent studies of fibronectin and laminin in the human eye. Invest Ophthalmol Vis Sci. 1987;28:506-514

170. Muraoka M, Hayashi T. Three polypeptides with distinct biochemical properties are major alpha chain-size components of type IV collagen in bovine lens capsule. J Biochem. 1993;114:358-362. doi:10.1093/oxfordjournals.jbchem.a124181

171. Brinker JM, Pegg MT, Howard PS, Kefalides NA. Immunochemical characterization of type IV procollagen from anterior lens capsule. Coll Relat Res. 1985;5:233-244. doi:10.1016/s0174-173x(85)80013-3

172. Kelley PB, Sado Y, Duncan MK. Collagen IV in the developing lens capsule. Matrix Biol. 2002;21:415-423. doi:10.1016/s0945-053x(02)00014-8

173. Marshall GE, Konstas AG, Bechrakis NE, Lee WR. An immunoelectron microscope study of the aged human lens capsule. Exp Eye Res. 1992;54:393-401. doi:10.1016/0014-4835(92)90051-s

174. Johnson DH, Bourne WM, Campbell RJ. The ultrastructure of Descemet's membrane. I. Changes with age in normal corneas. Arch Ophthalmol. 1982;100:1942-1947. doi:10.1001/archopht.1982.01030040922011

175. Sawada H. The fine structure of the bovine Descemet's membrane with special reference to biochemical nature. Cell Tissue Res. 1982;226:241-255. doi:10.1007/BF00218356

176. Thim K, Krag S, Corydon L. Stretching capacity of capsulorhexis and nucleus delivery. J Cataract Refract Surg. 1991;17:27-31. doi:10.1016/s0886-3350(13)80980-7

177. Hartmann U, Sistani F, Steinhorst UH. Human and porcine anterior lens capsule as support for growing and grafting retinal pigment epithelium and iris pigment epithelium. Graefes Arch Clin Exp Ophthalmol. 1999;237:940-945. doi:10.1007/s004170050390

178. Kozak I, Trbolova A, Zibrin M, Komorova T, Kolodzyeiski L, Juhas T. Electron microscopic study of anterior lens capsule allotransplants in chronic corneal ulcers. Cornea. 2004;23:797-803. doi:10.1097/01.ico.0000134195.71981.e2

179. Galal A, Perez-Santonja JJ, Rodriguez-Prats JL, Abad M, Alio J. Human anterior lens capsule as a biologic substrate for the ex vivo expansion of limbal stem cells in ocular surface reconstruction. Cornea. 2007;26:473-478. doi:10.1097/ICO. 0b013e318033bd0f

180. Spinozzi D, Miron A, Bruinsma M, Dapena I, Lavy I, Binder PS, Rafat M, Oellerich S, Melles GRJ. Evaluation of the Suitability of Biocompatible Carriers as Artificial Transplants Using Cultured Porcine Corneal Endothelial Cells. Curr Eye Res. 2019;44:243-249. doi:10.1080/02713683.2018.1536215

181. Telinius N, Spinozzi D, Rasic D, Dapena I, Baandrup U, Miron A, Oellerich S, Hjortdal J. Gottingen Minipig is not a Suitable Animal Model for in Vivo Testing of Tissue-Engineered Corneal Endothelial Cell-Carrier Sheets and for Endothelial Keratoplasty. Curr Eye Res.2020:1-5. doi:10.1080/02713683.2019.1706747

182. Fukuda K, Chikama T, Nakamura M, Nishida T. Differential distribution of subchains of the basement membrane components type IV collagen and laminin among the amniotic membrane, cornea, and conjunctiva. Cornea. 1999;18:73-79

183. Trelford JD, Trelford-Sauder M. The amnion in surgery, past and present. Am J Obstet Gynecol. 1979;134:833-845. doi:10.1016/0002-9378(79)90957-8

184. Kim JC, Tseng SC. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. Cornea. 1995;14:473-484

185. Chen HJ, Pires RT, Tseng SC. Amniotic membrane transplantation for severe neurotrophic corneal ulcers. Br J Ophthalmol. 2000;84:826-833. doi:10.1136/ bjo.84.8.826

186. Kubo M, Sonoda Y, Muramatsu R, Usui M. Immunogenicity of human amniotic membrane in experimental xenotransplantation. Invest Ophthalmol Vis Sci. 2001;42:1539-1546.

187. Utheim TP, Lyberg T, Raeder S. The culture of limbal epithelial cells. Methods Mol Biol. 2013;1014:103-129. doi:10.1007/978-1-62703-432-6_7

188. Suh JK, Matthew HW. Application of chitosan-based polysaccharide biomaterials in cartilage tissue engineering: a review. Biomaterials. 2000;21:2589-2598. doi:10.1016/s0142-9612(00)00126-5

189. Rafat M, Li F, Fagerholm P, Lagali NS, Watsky MA, Munger R, Matsuura T, Griffith M. PEG-stabilized carbodiimide crosslinked collagen-chitosan hydrogels for corneal tissue engineering. Biomaterials. 2008;29:3960-3972. doi:10.1016/j.biomaterials. 2008.06.017

190. Teichmann J, Valtink M, Nitschke M, Gramm S, Funk RH, Engelmann K, Werner C. Tissue engineering of the corneal endothelium: a review of carrier materials. J Funct Biomater. 2013;4:178-208. doi:10.3390/jfb4040178

191. Chattopadhyay S, Raines RT. Review collagen-based biomaterials for wound healing. Biopolymers. 2014;101:821-833. doi:10.1002/bip.22486

192. Jumblatt MM, Maurice DM, Schwartz BD. A gelatin membrane substrate for the transplantation of tissue cultured cells. Transplantation. 1980;29:498-499. doi:10.1097/00007890-198006000-00013

193. Yoshida J, Yokoo S, Oshikata-Miyazaki A, Amano S, Takezawa T, Yamagami S. Transplantation of Human Corneal Endothelial Cells Cultured on Bio-Engineered Collagen Vitrigel in a Rabbit Model of Corneal Endothelial Dysfunction. Curr Eye Res. 2017;42:1420-1425. doi:10.1080/02713683.2017.1351568

194. Sorushanova A, Delgado LM, Wu Z, Shologu N, Kshirsagar A, Raghunath R, Mullen AM, Bayon Y, Pandit A, Raghunath M, Zeugolis DI. The Collagen Suprafamily: From Biosynthesis to Advanced Biomaterial Development. Adv Mater. 2019 Jan;31(1):e1801651. doi: 10.1002/adma.201801651.

195. Schmidt MM, Dornelles RCP, Mello RO, Kubota EH, Mazutti MA, Kempka AP, Demiate IM. Collagen extraction process. Int Food Res J. 2016;23(3):913-922.

196. Wang T, Lew J, Premkumar J, Poh CL, Win Naing M. Production of recombinant collagen: state of the art and challenges. Eng Biol. 2017;1(1):18-23. doi: 10.1049/enb.2017.0003.

197. Liang Y, Liu W, Han B, Yang C, Ma Q, Zhao W, Rong M, Li H. Fabrication and characters of a corneal endothelial cells scaffold based on chitosan. J Mater Sci Mater Med. 2011;22:175-183. doi:10.1007/s10856-010-4190-6

198. Vazquez N, Chacon M, Meana A, Menendez-Menendez Y, Ferrero-Gutierrez A, Cereijo-Martin D, Naveiras M, Merayo-Lloves J. Keratin-chitosan membranes as scaffold for tissue engineering of human cornea. Histol Histopathol. 2015;30:813-821. doi:10.14670HH-11-585

199. Gao X, Liu W, Han B, Wei X, Yang C. Preparation and properties of a chitosan-based carrier of corneal endothelial cells. J Mater Sci Mater Med. 2008;19:3611-3619. doi:10.1007/s10856-008-3508-0

200. Vazquez N, Rodriguez-Barrientos CA, Aznar-Cervantes SD, Chacon M, Cenis JL, Riestra AC, Sanchez-Avila RM, Persinal M, Brea-Pastor A, Fernandez-Vega Cueto L, Meana A, Merayo-Lloves J. Silk Fibroin Films for Corneal Endothelial Regeneration: Transplant in a Rabbit Descemet Membrane Endothelial Keratoplasty. Invest Ophthalmol Vis Sci. 2017;58:3357-3365. doi:10.1167/iovs.17-21797

201. Salehi S, Czugala M, Stafiej P, Fathi M, Bahners T, Gutmann JS, Singer BB, Fuchsluger TA. Poly (glycerol sebacate)-poly (epsilon-caprolactone) blend nanofibrous scaffold as intrinsic bio- and immunocompatible system for corneal repair. Acta Biomater. 2017;50:370-380. doi:10.1016/j.actbio.2017.01.013

202. Kruse M, Walter P, Bauer B, Rutten S, Schaefer K, Plange N, Gries T, Jockenhoevel S, Fuest M. Electro-spun Membranes as Scaffolds for Human Corneal Endothelial Cells. Curr Eye Res. 2018;43:1-11. doi:10.1080/02713683.2017.1377258

203. Palchesko RN, Funderburgh JL, Feinberg AW. Engineered Basement Membranes for Regenerating the Corneal Endothelium. Adv Healthc Mater. 2016;5:2942-2950. doi:10.1002/adhm.201600488

204. Mimura T, Shimomura N, Usui T, Noda Y, Kaji Y, Yamgami S, Amano S, Miyata K, Araie M. Magnetic attraction of iron-endocytosed corneal endothelial cells to Descemet's membrane. Exp Eye Res. 2003;76:745-751. doi:10.1016/s0014-4835(03)00057-5

205. Mimura T, Yamagami S, Usui T, Ishii Y, Ono K, Yokoo S, Funatsu H, Araie M, Amano S. Long-term outcome of iron-endocytosing cultured corneal endothelial cell transplantation with magnetic attraction. Exp Eye Res. 2005;80:149-157. doi:10.1016/j.exer.2004.08.021

206. Mimura T, Yokoo S, Araie M, Amano S, Yamagami S. Treatment of rabbit bullous keratopathy with precursors derived from cultured human corneal endothelium. Invest Ophthalmol Vis Sci. 2005;46:3637-3644. doi:10.1167/iovs.05-0462

207. Mimura T, Yamagami S, Usui T, Seiichi, Honda N, Amano S. Necessary prone position time for human corneal endothelial precursor transplantation in a rabbit endothelial deficiency model. Curr Eye Res. 2007;32:617-623. doi:10.1080/02713680701530589

208. Patel SV, Bachman LA, Hann CR, Bahler CK, Fautsch MP. Human corneal endothelial cell transplantation in a human ex vivo model. Invest Ophthalmol Vis Sci. 2009;50:2123-2131. doi:10.1167/iovs.08-2653

209. Okumura N, Koizumi N, Ueno M, Sakamoto Y, Takahashi H, Tsuchiya H, Hamuro J, Kinoshita S. ROCK inhibitor converts corneal endothelial cells into a phenotype capable of regenerating in vivo endothelial tissue. Am J Pathol. 2012;181:268-277. doi:10.1016/j.ajpath.2012.03.033

210. Koizumi N, Okumura N, Ueno M, Nakagawa H, Hamuro J, Kinoshita S. Rho-associated kinase inhibitor eye drop treatment as a possible medical treatment for Fuchs corneal dystrophy. Cornea. 2013;32:1167-1170. doi:10.1097/ICO.0b013e318285475d

211. Okumura N, Ueno M, Koizumi N, Sakamoto Y, Hirata K, Hamuro J, Kinoshita S. Enhancement on primate corneal endothelial cell survival in vitro by a ROCK inhibitor. Invest Ophthalmol Vis Sci. 2009;50:3680-3687. doi:10.1167/iovs.08-2634

212. Okumura N, Koizumi N, Ueno M, Sakamoto Y, Takahashi H, Hamuro J, Kinoshita S. The new therapeutic concept of using a rho kinase inhibitor for the treatment of corneal endothelial dysfunction. Cornea. 2011;30 Suppl 1:S54-59. doi:10.1097/ICO.0b013e3182281ee1

213. Okumura N, Kakutani K, Inoue R, Matsumoto D, Shimada T, Nakahara M, Kiyanagi Y, Itoh T, Koizumi N. Generation and Feasibility Assessment of a New Vehicle for Cell-Based Therapy for Treating Corneal Endothelial Dysfunction. PLoS One. 2016;11:e0158427. doi:10.1371/journal.pone.0158427

214. Okumura N, Matsumoto D, Fukui Y, Teramoto M, Imai H, Kurosawa T, Shimada T, Kruse F, Schlotzer-Schrehardt U, Kinoshita S, Koizumi N. Feasibility of cell-based therapy combined with descemetorhexis for treating Fuchs endothelial corneal dystrophy in rabbit model. PLoS One. 2018;13:e0191306. doi:10.1371/journal.pone.0191306

215. Peh GSL, Ong HS, Adnan K, Ang HP, Lwin CN, Seah XY, Lin SJ, Mehta JS. Functional Evaluation of Two Corneal Endothelial Cell-Based Therapies: Tissue-Engineered Construct and Cell Injection. Sci Rep. 2019;9:6087. doi:10.1038/s41598-019-42493-3

216. Jia Y, Li W, Duan H, Li Z, Zhou Q, Shi W. Mini-Sheet Injection for Cultured Corneal Endothelial Transplantation. Tissue Eng Part C Methods. 2018;24:474-479. doi:10.1089/ten.TEC.2018.0077

217. Ong HS, Peh G, Neo DJH, Ang HP, Adnan K, Nyein CL, Morales-Wong F, Bhogal M, Kocaba V, Mehta JS. A Novel Approach of Harvesting Viable Single Cells from Donor Corneal Endothelium for Cell-Injection Therapy. Cells. 2020;9. doi:10.3390/cells9061428

218. Okumura N, Kinoshita S, Koizumi N. Application of Rho Kinase Inhibitors for the Treatment of Corneal Endothelial Diseases. J Ophthalmol. 2017;2017:2646904. doi:10.1155/2017/2646904

219. Dana R. A New Frontier in Curing Corneal Blindness. N Engl J Med. 2018;378:1057-1058. doi:10.1056/NEJMe1800726

220. Parikumar P, Haraguchi K, Senthilkumar R, Abraham SJ. Human corneal endothelial cell transplantation using nanocomposite gel sheet in bullous keratopathy. Am J Stem Cells. 2018;7:18-24.

221. Parikumar P, Haraguchi K, Ohbayashi A, Senthilkumar R, Abraham SJ. Successful transplantation of in vitro expanded human cadaver corneal endothelial precursor cells on to a cadaver bovine's eye using a nanocomposite gel sheet. Curr Eye Res. 2014;39:522-526. doi:10.3109/02713683.2013.838633

222. European Parliament. Regulation (EC) No 1394/2007 and an amendment to Directive 2001/83/EC.

223. Ziegele B, Dahl L, Muller AT. [The Innovation Office of the Paul-Ehrlich-Institut. Regulatory support during the scientific development of ATMP]. Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz. 2011;54:857-866. doi:10.1007/s00103-011-1307-0

224. Fujita Y, Kawamoto A. Regenerative medicine legislation in Japan for fast provision of cell therapy products. Clin Pharmacol Ther. 2016;99:26-29. doi:10.1002/cpt.279

225. U.S. FDA: Combination product definition. Content current as of 15 Feb 2018. https://www.fda.gov/combination-products/about-combination-products/combination-product-definition-combination-product-types.

226. Detela G, Lodge A. EU Regulatory Pathways for ATMPs: Standard, Accelerated and Adaptive Pathways to Marketing Authorisation. Mol Ther Methods Clin Dev. 2019;13:205-232. doi:10.1016/j.omtm.2019.01.010

227. Iglesias-Lopez C, Agusti A, Obach M, Vallano A. Regulatory Framework for Advanced Therapy Medicinal Products in Europe and United States. Front Pharmacol. 2019;10:921. doi:10.3389/fphar.2019.00921

228. U.S. FDA: Tissue & tissue products. Content current as of 11 Jul 2019. https://www.fda.gov/vaccines-blood-biologics/tissue-tissue-products.

229. McCarthy RC, Breite AG, Green ML, Dwulet FE. Tissue dissociation enzymes for isolating human islets for transplantation: factors to consider in setting enzyme acceptance criteria. Transplantation. 2011;91:137-145. doi:10.1097/TP.0b013e3181ffff7d

230. Bond MD, Van Wart HE. Characterization of the individual collagenases from Clostridium histolyticum. Biochemistry. 1984;23:3085-3091. doi:10.1021/bi00308a036

231. Marcus GJ, Connor L, Domingo MT, Tsang BK, Downey BR, Ainsworth L. Enzymatic dissociation of ovarian and uterine tissues. Endocr Res. 1984;10:151-162. doi:10.3109/07435808409035415

232. Ray JL, Leach R, Herbert JM, Benson M. Isolation of vascular smooth muscle cells from a single murine aorta. Methods Cell Sci. 2001;23:185-188. doi:10.1023/a:1016357510143

233. Szabo Z, Harasztosi C, Szucs G, Sziklai I, Rusznak Z. A detailed procedure and dissection guide for the isolation of spiral ganglion cells of the guinea pig for electrophysiological experiments. Brain Res Brain Res Protoc. 2003;10:139-147. doi:10.1016/s1385-299x(02)00205-2

234. Li Y, Wang Y, Wu Q, Li L, Shi Y, Bu H, Bao J. Comparison of methods for isolating primary hepatocytes from mini pigs. Xenotransplantation. 2016;23:414-420. doi:10.1111/xen.12259

235. Raposio E, Simonacci F, Perrotta RE. Adipose-derived stem cells: Comparison between two methods of isolation for clinical applications. Ann Med Surg (Lond). 2017;20:87-91. doi:10.1016/j.amsu.2017.07.018

236. Vianna LM, Kallay L, Toyono T, Belfort R, Jr., Holiman JD, Jun AS. Use of human serum for human corneal endothelial cell culture. Br J Ophthalmol. 2015;99:267-271. doi:10.1136/bjophthalmol-2014-306034

237. Thieme D, Reuland L, Lindl T, Kruse F, Fuchsluger T. Optimized human platelet lysate as novel basis for a serum-, xeno-, and additive-free corneal endothelial cell and tissue culture. J Tissue Eng Regen Med. 2018;12:557-564. doi:10.1002/term.2574

238. Toda M, Ueno M, Hiraga A, Asada K, Montoya M, Sotozono C, Kinoshita S, Hamuro J. Production of Homogeneous Cultured Human Corneal Endothelial Cells Indispensable for Innovative Cell Therapy. Invest Ophthalmol Vis Sci. 2017;58:2011-2020. doi:10.1167/iovs.16-20703

239. Bartakova A, Alvarez-Delfin K, Weisman AD, Salero E, Raffa GA, Merkhofer RM, Jr., Kunzevitzky NJ, Goldberg JL. Novel Identity and Functional Markers for Human Corneal Endothelial Cells. Invest Ophthalmol Vis Sci. 2016;57:2749-2762. doi: 10.1167/iovs.15-18826

240. Frausto RF, Le DJ, Aldave AJ. Transcriptomic Analysis of Cultured Corneal Endothelial Cells as a Validation for Their Use in Cell Replacement Therapy. Cell Transplant. 2016;25:1159-1176. doi:10.3727/096368915X688948

241. Ueno M, Asada K, Toda M, Nagata K, Sotozono C, Kosaka N, Ochiya T, Kinoshita S, Hamuro J. Concomitant Evaluation of a Panel of Exosome Proteins and MiRs for Qualification of Cultured Human Corneal Endothelial Cells. Invest Ophthalmol Vis Sci. 2016;57:4393-4402. doi:10.1167/iovs.16-19805

242. Ueno M, Asada K, Toda M, Schlotzer-Schrehardt U, Nagata K, Montoya M, Sotozono C, Kinoshita S, Hamuro J. Gene Signature-Based Development of ELISA Assays for Reproducible Qualification of Cultured Human Corneal Endothelial Cells. Invest Ophthalmol Vis Sci. 2016;57:4295-4305. doi:10.1167/iovs.16-19806

243. Ueno M, Asada K, Toda M, Hiraga A, Montoya M, Sotozono C, Kinoshita S, Hamuro J. MicroRNA Profiles Qualify Phenotypic Features of Cultured Human Corneal Endothelial Cells. Invest Ophthalmol Vis Sci. 2016;57:5509-5517. doi:10.1167/iovs.16-19804

244. Winton HL, Bidwell JL, Armitage WJ. Functional tumor necrosis factor alpha polymorphisms and haplotype analysis in high-risk corneal transplantation. Transplant Proc. 2014;46:1548-1553. doi:10.1016/j.transproceed.2014.02.029

245. Winton HL, Bidwell JL, Armitage WJ. Haplotype analysis on chromosome 6p of tumor necrosis factor alpha, vascular endothelial growth factor A, and interleukin-17F alleles associated with corneal transplant rejection. Transplant Proc. 2014;46:1540-1547. doi:10.1016/j.transproceed.2014.04.003

246. van Essen TH, Roelen DL, Williams KA, Jager MJ. Matching for Human Leukocyte Antigens (HLA) in corneal transplantation - to do or not to do. Prog Retin Eye Res. 2015;46:84-110. doi:10.1016/j.preteyeres.2015.01.001

247. Yamada J, Ueno M, Toda M, Shinomiya K, Sotozono C, Kinoshita S, Hamuro J. Allogeneic Sensitization and Tolerance Induction After Corneal Endothelial Cell Transplantation in Mice. Invest Ophthalmol Vis Sci. 2016;57:4572-4580. doi:10.1167/iovs.15-19020

248. Dragnea DC, Ni Dhubhghaill S, Bourgonje VJA, Dapena I, Oellerich S, Melles GRJ, Lavy I. In Vitro Evaluation of the Feasibility of Slit-Lamp Nd: YAG Laser Descemetorhexis. Cornea. 2020;39:229-233. doi:10.1097/ICO.0000000000002109

249. Kassumeh S, von Studnitz A, Priglinger SG, Fuchshofer R, Luft N, Moloney G, Dirisamer M, Ohlmann A. Ex vivo excimer laser ablation of cornea guttata and ROCK inhibitor-aided endothelial recolonization of ablated central cornea. Acta Ophthalmol. 2020. doi:10.1111/aos.14366

250. Mehta JS, Kocaba V, Soh YQ. The future of keratoplasty: cell-based therapy, regenerative medicine, bioengineering keratoplasty, gene therapy. Curr Opin Ophthalmol. 2019;30:286-291. doi:10.1097/ICU.0000000000000573

251. Soh YQ, Peh GS, Mehta JS. Evolving therapies for Fuchs' endothelial dystrophy. Regen Med. 2018;13:97-115. doi:10.2217/rme-2017-0081