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Focus on cell therapy to treat corneal endothelial diseases

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Abbreviation

AQP: AQuaPorins
ATP: Adenosine TriPhosphate
bFGF: basic Fibroblast Growth Factors
CEncs: Corneal Endothelial Cells
CK: cytokeratin
DM: Descemet's Membrane
DMEK: Descemet Membrane Endothelial Keratoplasty
DXM: Dexamethasone
ECD: Endothelial Cell Density
ECM: ExtraCellular Matrix
EDTA: EthyleneDiamineTetraAcetic acid
FACS: Fluorescence Activated Cell Sorting
FBS: Fetal Bovine Serum
FECD: Fuchs Endothelial Corneal Dystrophy
FNC: Fibronectin Collagen and albumin
HCO₃⁻: Bicarbonate
iPSC: Induced Pluripotent Stem Cell
K⁺: Potassium
LC: Lens Capsule
LCSCs: Limbal Corneal Stem Cells
MACS: Magnetic Activated Cell Sorting
Mg²⁺: Magnesium
MSC: Mesenchymal Stem Cell
Na⁺: Sodium
PBMC: Peripheral Blood Monocyte Cells
PIPAAm: Polymer PolyN-Isopropylacrylamide
SdFFF: Sedimentation Field-Flow Fractionation
SKP: SKin-derived Precursors
TGF-beta: Transforming Growth Factor beta
VEGF: Vascular Endothelial Growth Factor
ZO-1: zonula occludens-1

Abstract

The cornea is a multi-layered structure which allows fine refraction and provides both resistance to external insults and adequate transparency. The corneal endothelium ensures stromal hydration, failure of which, such as in Fuchs endothelial corneal dystrophy, after trauma or in aging, may lead to loss of corneal transparency and induce blindness. Currently, no efficient therapeutic alternatives exist except for corneal grafting. Thus corneal tissue engineering represents a valuable alternative approach, which may overcome cornea donor shortage. Several studies describe protocols to isolate, differentiate, and cultivate corneal endothelial cells (CEnCs) *in vitro*. Two main *in vitro* strategies can be described: expansion of eye-native cell populations, such as CEnCs, or the production and expansion of CEnCs from non-eye native cell populations, such as induced Pluripotent Stem Cells (iPSC). The challenge with these cells is to obtain a monolayer of CEnCs on a biocompatible carrier, with a specific morphology (flat hexagonal cells), and with specific functions such as programmed cell cycle arrest. Another issue for this cell culture methodology is to define the adapted protocol (media, trophic factors, timeframe) that can mimic physiological development. Additionally, contamination by other cell types still represents a huge problem. Thus, purification methods, such as Fluorescence Activated Cell Sorting (FACS), Magnetic Activated Cell Sorting (MACS) or Sedimentation Field Flow Fractionation (SdFFF) are useful. Animal models are also crucial to provide a translational approach for these therapies, integrating macro- and microenvironment influences, systemic hormonal or immune responses, and exogenous interactions. Non-eye native cell graft protocols are constantly improving both in efficacy and safety, with the aim of being the most suitable candidate for corneal therapies in future routine practice. The aim of this work is to review these different aspects with a special focus on issues facing CEnC culture *in vitro*, and to highlight animal graft models adapted to screen the efficacy of these different protocols.

Keywords

Cornea; corneal endothelial cells; CEnCs; cellular therapy; induced pluripotent stem cells; graft; animal models.

Introduction

Vision is fully dependent on a transparent cornea, which is essential for the formation of a focused image on the retina. The human cornea is arranged into well-organized layers, and each layer plays a significant role in maintaining the transparency and viability of the tissue. The corneal endothelium consists of a monolayer of flattened cells facing the anterior chamber of the eye. Its main role is to maintain corneal transparency by ensuring stromal dehydration, a process achieved through ionic pumps and ion transporters (Van den Bogerd et al., 2018a). Human corneal endothelial cells are believed to be unable to proliferate *in-vivo* and are arrested in the G1-phase of the cell cycle (Kim et al., 2001). If cells degenerate, neighboring cells become enlarged and migrate in order to maintain the integrity of the monolayer (Joko et al., 2017). The consequence of this poor cell density is a lack of stromal dehydration and the loss of corneal transparency, which leads to blindness. This endothelial failure can result from several conditions such as Fuchs endothelial corneal dystrophy (FECD) or from surgical trauma secondary to cataract surgery. There are currently no efficient therapeutic alternatives to corneal transplantation for corneal endothelial failure. In 2014, the Eye Bank Association of America reported that more than 40% of all US corneal grafts were used to treat corneal endothelial diseases but that the shortage of donors limits these transplantations to only 1 in 70 potential patients worldwide (Gain et al., 2016). Although the estimated risk of a first immune rejection episode after endothelial graft is only around 7.6% during the first year, and 12% during the second year, this process results in a significant progressive endothelial cell loss (Li et al., 2012; Price et al., 2009).

Corneal tissue engineering represents an alternative to heterologous corneal transplantation and represents a means of overcoming the cornea donor shortage (Mimura et al., 2012). Additionally, generating a new and highly functional corneal endothelium produced from eye-native corneal cell expansion or from autologous cells, such as Skin-derived precursors (SKP), Mesenchymal Stem Cells (MSCs) or Induced Pluripotent Stem Cells (iPSCs), would limit immune rejection. However, the ideal tissue-engineered cornea needs to be nontoxic, free of transmissible diseases, be genetically stable, immunologically compatible and have a stable long-term function and high cell survival rate. It also needs to be compatible with current surgical techniques for implantation. Tissue engineering procedures consist of several crucial steps, including tissue removal, cell isolation, cell culture and implantation via an adapted carrier (Shah et al., 2008). This review focuses on different cell therapy strategies adapted to endothelial corneal diseases according to tissue engineering procedures and surgical practices, and also on the animal models adapted to test the efficacy of these procedures.

1. Anatomy and physiology of the human corneal endothelium

The cornea is classically composed of five distinguishable layers. Three of these are cellular (epithelium, stroma and endothelium), while two are acellular (Bowman's and Descemet's membranes) (Fig. 1). The corneal endothelium plays a critical role in maintaining a relatively dehydrated state of the stroma, preserving the precise organization of stromal collagen fibers and, in turn, corneal transparency. Any distortion of this corneal transparency induces optical aberrations and results in decreased vision quality (DelMonte and Kim, 2011).

Around the sixth week of gestation, corneal endothelial cells (CEnCs), originating from the neural crest (neuroectoderm), migrate centrally from the rim of the optic cup to form a monolayer of cuboidal cells. Over time, these cells flatten and begin to secrete their basal membrane, termed Descemet's membrane (DM), located between the stroma and the endothelium. DM is composed of

two regular layers of collagen type IV, VIII and XII, and laminin (Kabosova et al., 2007). At birth, the endothelium is a uniform monolayer about 10 μm in thickness and consisting of around 350,000 cells ($\sim 6,000$ cells/ mm^2), covering the posterior surface of the cornea and fusing with the cells of the trabecular meshwork at the periphery. After birth, endothelial cells continue to flatten, stabilizing at approximately 4 to 6 μm thickness in adulthood. They appear as a uniform honeycomb-like mosaic with four to nine sides, about 20 μm in diameter and 250 μm^2 in cell area. This specific hexagonal morphology is easily seen in clinical practice using specular microscopy (Fig. 1). Adjacent cells interdigitate, overlap and form gaps and tight junctions at their lateral borders. The lateral membranes possess a high density of Na^+/K^+ -ATPase pump sites. However, endothelial cell morphometry continues to change throughout life. At birth, endothelial cell density (ECD) is around 6,000 cells/ mm^2 . Afterwards, during infancy, as the corneal surface expands, ECD dramatically decreases to a value around 3,500 cells/ mm^2 . Thereafter, from the second to ninth decades of life, the cell density decreases slowly from 3,300 cells/ mm^2 to 2,300 cells/ mm^2 , with an increase in cell area from 290 to 450 μm^2 . The coefficient of variation of mean cell area (polymegethism) increases from 0.22 to 0.29 and the percentage of hexagonal cells decreases from 75% to 60% (pleomorphism), indicating that individual endothelial cells become less uniform throughout life (Yee et al., 1985). The intercellular space between endothelial cells measures from 25 to 40 nm. This space decreases to about 3 nm at the apical borders of the cells, where junctional complexes exist. These junctional complexes consist of focal tight junctions (zonulae occludens) and gap junctions (Waring et al., 1982).

The corneal endothelium maintains corneal transparency by ensuring corneal deturgescence, a condition in which the corneal stroma is relatively dehydrated (being around 78% water content). This process is achieved through the actions of ionic pumps (Na^+/K^+ -ATPase) and ion transporters such as the co-transporter $\text{Na}^+/\text{HCO}_3^-$, while aquaporins (AQP) facilitate the flow of water in response to osmotic gradients created by those pumps (Verkman et al., 2008). The corneal stroma imbibes aqueous fluids due to the presence of glycosaminoglycans, which results in fluid from the aqueous humor being pulled into the stroma, while the corneal endothelium actively pumps the fluid

out of the stroma (pump function and AQP of the endothelium). Under physiological conditions, the leaky barrier of the endothelium equals its pump function. In conditions of imbalance, there is excessive fluid diffusion into the stroma, disrupting the uniform arrangement of collagen fibers and resulting in light scattering and corneal opacity. The Na⁺/K⁺-ATPase pumps actively exchange 3 sodium ions from inside the cell with 2 potassium ions from outside the cell. This pump is magnesium dependent and requires ATP. Many mitochondria are located in this area, close to the cytoplasmic membrane to provide the ATP necessary for maintenance of the pump function. Although endothelial cell density declines throughout life, the density of Na⁺/K⁺-ATPase pumps on the corneal endothelium remains constant regardless of age, suggesting that the remaining endothelial cells adapt to cell loss by expressing new pump sites in order to maintain normal pump site density and thus maintain pump capacity (Geroski et al., 1985). The activity of these endothelial pumps can be affected by a number of conditions that cause corneal swelling, such as lowering of temperature, or exposure to bicarbonate-free solutions (Waring et al., 1982). In addition to the Na⁺/K⁺-ATPase pump, AQP water channels, proteins that are expressed in the corneal endothelium, facilitate transmembrane solute-free water transport in response to osmotic gradients. Deficiency in this significantly delays restoration of corneal transparency and thickness. However, the mechanism involved here remains unclear (Verkman et al., 2008).

2. Classical therapeutic approaches for corneal endothelial dysfunction

The most common primary corneal endothelial dysfunction is FECD (3.8% to 11% in patients older than 40 years (Darlington et al., 2006)), characterized by a progressive decrease in endothelial cell count, alterations in shape and size of the residual cells, and formation of guttae (Darlington et al., 2006; Higa et al., 2011). As the disease progresses, endothelial cell density decreases until the residual cells are no longer able to maintain corneal deturgescence, resulting in corneal clouding and decreased vision (Schmedt et al., 2012). Keratoplasty is the standard surgical treatment for patients with corneal decompensation which results in good visual recovery. However, in eyes where there is

poor visual potential (early stages of FECD), or when donor tissue is not available for keratoplasty, other approaches should be considered to reduce pain and discomfort. Topical 5% hypertonic sodium chloride (eye drops and ointment) is useful in the early stage of corneal decompensation, to reduce corneal thickness and improve visual acuity, but it is not effective in the advanced stage of the disease (Knezović et al., 2006). Moreover, some patients cannot tolerate such eye drops because of pain linked to ocular irritation. Silicone hydrogel bandage contact lenses can also be used, alone or in combination with hypertonic saline eye drops, to alleviate pain and discomfort (Lim and Vogt, 2006). The standard treatment for moderate-to-severe endothelial cell decompensation is to surgically replace these with viable donor cells. Eduard Zirmin, in 1905, performed the first successful keratoplasty in which the graft remained clear (Moffatt et al., 2005). The graft was derived from a human eye that was enucleated directly prior to full-thickness penetrating keratoplasty (PK), the graft being secured with overlay sutures. Thus, PK has been the standard keratoplasty technique used to replace degenerating or lost endothelium since corneal grafting became a routine operation in the 1950s. Although penetrating keratoplasty provides good visual outcomes, it may be complicated by prolonged recovery due to persistent epithelial defects, complications with sutures and wound leak, giant astigmatism, endophthalmitis, primary endothelial failure microbial keratitis, primary disease recurrence and immune rejection.

In the past decade, novel minimally-invasive lamellar keratoplasty techniques for a variety of corneal diseases have been developed, which generally result in better outcomes when compared to full-thickness PK. This has led to fundamental changes in corneal transplantation, and the majority of keratoplasties can now be performed in a lamellar fashion. Endothelial keratoplasty (EK) procedures are comprised of the selective replacement of Descemet membrane and the adherent corneal endothelial cell (CEC) layer. The most popular techniques for posterior lamellar endothelial keratoplasty (EK) currently are Descemet stripping automated endothelial keratoplasty (DSAEK) and Descemet Membrane Endothelial Keratoplasty (DMEK), the latter introduced by Melles (Melles et al., 2006).

Partial-thickness endothelial keratoplasty with selective replacement of the endothelium significantly reduces preoperative and postoperative risks, as well as improving time to recovery (Price et al., 2009). These techniques maintain biomechanical stability of the cornea better than penetrating keratoplasty as complete corneal trepanation can be avoided. Another significant advantage of avoiding trepanation is that surgery can also be performed under local anesthesia avoiding an “open sky” situation. Moreover, only few or no sutures are used to close the surgical incisions. Important benefits of EK compared to PK include a better functional outcome, faster visual recovery, reduced risk of hemorrhage and infection, less astigmatism, less corneal denervation, a tectonically more stable result, less vessel growth, and fewer episodes of graft rejection (Anshu et al., 2012).

Despite all these advantages, the visual result in some patients may be less than optimal even with fully functional grafts and no ocular comorbidities, suggesting that there is a ceiling for what can be achieved in some cases (Turnbull et al., 2016). Furthermore, not all corneal transplantations can be performed in a lamellar fashion, due to these novel techniques not being available worldwide, and there are still corneal pathologies that require the replacement of all corneal layers. In these cases, immune-mediated endothelial graft rejection is highly relevant, especially in pathologically vascularized high-risk eyes which constitute around 10% of all grafts, meaning currently available treatment options remain unsatisfactory.

3. Cell therapy in the field of endothelial corneal diseases

As mentioned above, shortage of corneal grafts is a significant and unmanageable limit on corneal transplantations. Thus, cellular therapy is considered an emerging field of research since it provides, theoretically, an unlimited source of endothelial corneal tissues while limiting immune rejection. Based on the abundant literature on cell and tissue engineering, cell therapy has the potential to replace deficient tissues of the cornea using autologous or heterologous cells (Navaratnam et al., 2015; Proulx and Brunette, 2012; Wagoner et al., 2018).

3.1. Different sources of corneal endothelial cells

Different cell lines may be suitable for corneal endothelial cell therapies. Several studies have described *in vitro* protocols to isolate, differentiate, and cultivate corneal endothelial cells (CEnCs). Two main strategies can be distinguished *in vitro*: expansion of eye-native cell populations such as CEnCs or production and expansion of CEnCs from non-eye native cell populations.

Eye-native corneal cell expansion. This consists of expanding *in vitro* eye-native cells before transplanting them back into the affected/lacking eye. Two eye-native cell populations may be useful: CEnCs from corneal endothelial stem cell differentiation and CEnCs obtained by direct expansion (Hsu et al., 2015). A stem cell differentiation strategy has already been developed for corneal epithelial therapies using Limbal corneal stem cells (LCSCs). The LCSCs are *in vivo* corneal stem cell population found in the limbal cornea at the corneo-scleral junction and are involved in constant corneal epithelial regeneration. To our knowledge, LCSCs have never been shown to be able to regenerate the corneal endothelium and their deficiency has not been shown to cause corneal endothelial dysfunction (Gonzalez et al., 2018; Saghizadeh et al., 2017). LCSCs cannot be considered as a real candidate yet because of their limited proliferative capacity in response to massive endothelial damage (He et al., 2012), and the lack of available markers to clearly identify and isolate them. It is also hypothesized that an endothelial stem cell population could exist in the peripheral corneal endothelium and can migrate from the periphery to the center of the corneal endothelium (He et al., 2012). The second strategy is based on *in vitro* expansion of CEnCs. Protocols based on extraction of CEnCs from human corneal grafts already exist (Proulx and Brunette, 2012). The most common technique consists of peeling the DM from the corneal graft before digesting it with collagenase, dispase or ethylene diamine tetra acetic acid (EDTA) (Peh et al., 2011). The remaining cells are then cultured *in vitro* in specific culture media. The first issue with this technique is contamination by keratocyte cells (from the epithelial layer) due to their higher proliferative capacities compared with CEnCs. Secondly, CEnCs *in vitro* show poorer proliferation compared with the situation *in vivo* (Peh et al., 2011), especially when derived from older donors (Zhao and Afshari, 2016). Human CEnCs cultivated on bovine extra-cellular matrix (bovine atelocollagen in basal

medium with bFGF and L-ascorbic acid 2-phosphate) appear to show better proliferation compared to cells grown using standard protocols (Shima et al., 2011). Sequential stimulation or inhibition of the TGF-beta pathway has been reported to enhance *in vitro* CEnCs development. Firstly, TGF-beta inhibition (by SMAD7 or SB431542) may stimulate CEnCs proliferation and reduce retrocorneal fibrous membrane formation (Saghizadeh et al., 2017). Once CEnCs reach confluence, supplementation with TGF-beta seems to induce an endothelial morphology, including lateral localization of zonula occludens-1 (ZO-1) and acquisition of pumping functions (Beaulieu Leclerc et al., 2018). However, this strategy had several limitations. Peel-and-digest method must be used on research-grade human corneas to obtain CEnCs. In addition, these cells need to be isolated from several transplants and mixed to have a good quantity of cells improving proliferation rate (Peh et al., 2011). In addition, the successful of CEnCs culture is linked to specific culture conditions based on optimized medium supplementation (Frausto et al., 2020; Spinozzi et al., 2018) with a high donor/recipient ratio (1:5 to 1:15) and a cell density around 3,000 cells/mm² (Frausto et al., 2020). Regarding culture medium components, they included animal products like fetal bovine serum, so their use is limited to experimental research. Finally, the risk of allograft rejection always remained.

Non-eye native stem-cell based therapy. Autologous stem cell-based therapies, offering immune tolerance, could provide benefits both in terms of quality of life and medical cost-reduction. Since the use of embryonic stem cells raises obvious ethical issues, this review will only focus on autologous, easily accessible adult stem cells: these include Skin-derived Precursors (SKP), MSCs (Mesenchymal Stem Cells) and Induced Pluripotent Stem Cells (iPSCs).

Because cell reprogramming-differentiation mimics successive embryonic developmental steps, the issues with these procedures are that both mechanisms of CEnC embryology and differentiation pathways remain unclear. As previously described, corneal epithelial cells differentiate from the superficial ectoderm whereas CEnCs may be derived from neural-crest cells (Gonzalez et al., 2018). Generally, multiple differentiation steps have been hypothesized. Firstly, embryonic pluripotent mesenchymal stem cells could be directed towards an ocular differentiation phenotype through

inhibition of SMAD and Wnt/beta catenin pathways. Secondly, activation of Wnt pathway permits differentiation towards a neural-crest cell fate (Nuzzi et al., 2018; Zhao and Afshari, 2016). Finally, inhibition of TGF-beta and ROCK pathways may further guide differentiation towards a corneal fate (Zhao and Afshari, 2016). However, some authors have suggested that CEnCs may rather originate from a different specific cell population: ocular specific neural-crest cells (Wagoner et al., 2018). Associated with these embryological characteristics, several protocols have been described to produce CEnCs according to the stem cell origin, including from Peripheral Blood Monocyte Cells (PBMC), iPSC or fibroblasts (Ali et al., 2018; Nuzzi et al., 2018).

Only a few published studies have focused on SKPs obtained from embryonic and adult skin and then differentiated into multiples cells types (Dai et al., 2018), including corneal endothelial cells that were grafted in mouse or rabbit models (Inagaki et al., 2017).

On the other hand, MSCs do possess several advantages: they are easily accessed in adults (adipose tissue, bone-marrow, skeletal muscle etc.), they raise limited ethical issues, and have low potential for cancerogenesis (Castro-Manrreza and Montesinos, 2015). This explains why 350 clinical trials using MSCs in corneal endothelium therapies are presently underway (Gutermuth et al., 2019). However, MSCs from adult tissues also present issues including heterogenous proliferative and differentiation capacities, with differences in morphological results seen after differentiation, thus hindering the development of a standard protocol (Nuzzi et al., 2018).

Lastly, iPSC technology is an innovative and efficient tool in obtaining CEnCs, since it can theoretically be both autologous and also adapted to all corneal cell types (Takahashi and Yamanaka, 2006) (Fig.2). Table 1 summarizes several differentiation protocols that are both feeder-free and serum-free in obtaining CEnCs from iPSCs (mimicking embryogenic differentiation). These published data underlined differences in the timing of supplementation, the use of inhibitors of different signaling pathways, culture density and time of passage, showing heterogenous efficiency (Ali et al., 2018). After differentiation and proliferation steps, the critical step in cell therapy is to then isolate and purify the cells of interest.

3.2. Corneal endothelial cell characterization, isolation, and purification

Most isolation processes are antibodies-based techniques requiring detection by fluorescence or by coupling with magnetic particles. However, a tagless technique, called Sedimentation Field Flow Fractionation (SdFFF) allows isolating cells without the use of antibodies (Battu et al., 2002).

The first of these techniques require specific markers of CEnCs. These cells express, in particular, vimentin, S100 protein, neuron-specific-enolase (Hayashi et al., 1986) and cytokeratin (CK) markers including CK8 and CK18, but are negative for CK1, CK3/12, CK4, CK5/6, CK7, CK10, CK10/13, CK14, CK16, CK17, and CK19 (Jirsova et al., 2007). They also express the cytoplasmic complex ZO-1, which is associated with tight junctions, the Dickkopf Wnt signaling pathway inhibitor 1 during embryogenesis (Chen et al., 2016), aquaporin-1 (but no other aquaporins), and thrombomodulin, also known as CD141 or BDCA-3 (Fischbarg et al., 2006). CEnCs also express alpha-catenin, beta-catenin, lakoglobulin, and the gap junction protein connexin-43 (Eghrari et al., 2015).

Many researchers have already employed FACS to purify cell cultures using antibodies against CEnCs markers (Hargus et al., 2010). The main issue here is to determine the best marker of CEnCs. Indeed, markers such as ZO-1, Na⁺/K⁺-ATPase are present on CEnCs, but are also found on other cell populations, making them unsuitable for CEnCs isolation (Navaratnam et al., 2015). Similarly, an anti-mouse Flk1 antibody combined with an anti-mouse VE-cadherin may also be useful for CEnCs isolation but these markers are also found in all cells of endothelial origin (Chatterjee et al., 2016). Moreover, Flk1 (also known as VEGFR-2), is expressed in adult neurons and astrocytes, making the Flk1 marker of questionable utility for the isolation of CEnCs from neural crest cells (Harris et al., 2017). Another method which has been used to isolate CEnCs is MACS, in which magnetic nanoparticles coated with specific antibodies are used to select the cell population of interest (Leng et al., 2019). Unlike FACS, this technique is more rapid, but many antibodies are unavailable. Overall, all of these techniques have some weaknesses, such as antibody availability, interference of the antibody with later cell use, including implantation in the eye, or they may promote cell dedifferentiation (Diogo et al., 2012).

SdFFF may represent a good alternative method. This is a cell sorting method based on the intrinsic biophysical properties of cells such as their stiffness, deformability, size and density. SdFFF is a preparative, comparatively gentle and passive method when compared to the methods previously detailed (Battu et al., 2002). Using this non-immunostaining technique, we previously showed that embryoid bodies derived from iPSCs could be sorted into neural progenitor or endothelial progenitor cells, without the use of any specific media or trophic factors (Faye et al., 2016). It may therefore be interesting to employ this technique in order to obtain an enriched endothelial population as is expected for CEnCs.

4.3 Cell support for *in vivo* implantation.

While *in vitro* expansion of CEnCs appears to be adequately managed through these different approaches, the cell or reconstructed tissue reimplantation process seems to be the limiting step of this therapy. The main problem is that *in vitro* produced tissues are very thin and thus difficult to manipulate without damaging them during *in vivo* implantation into the recipient. According to literature, a lot of carriers could be used for cornea grafting such as amniotic membrane (Ishino et al., 2004), natural polymers (collagen 1 and 4, laminin, fibronectin, gelatin, association of laminin and chondroitin sulfate, combination of fibronectin collagen and albumin (Navaratnam et al., 2015)), anterior crystalline lens capsule (LC) (Van den Bogerd et al., 2018b), silk fibroin (Higa et al., 2011), polyethylene glycol, Polymer poly(N-isopropylacrylamide), stroma, or DM (Navaratnam et al., 2015).

Cell carrier issues and perspectives. *In vivo*, CEnCs grow attached to DM which is synthesized by CEnCs themselves (Johnson et al., 1982). This support needs to promote CEnCs adherence and also enhance CEnCs DM production, leading to transparency of the stroma after transplantation. An extensive review describing the different carriers used in this strategy was published in 2015 (Navaratnam et al., 2015). In this review we focus our attention on decellularization processes adapted to *in vivo* tissue (Table 2). Briefly, different processes exist for producing carriers, consisting essentially of either mechanical or chemical techniques. Mechanical scraping of the corneal endothelium is feasible (Navaratnam et al., 2015). On the other hand, freeze-thaw protocols can

maintain biochemical aspects of the tissue but are ineffective in terms of decellularization. The use of ethanol combined with various levels of CO₂ exposure, temperature and pressure, and supercritical carbon dioxide techniques succeed in maintaining the native form of extracellular matrix (ECM) proteins and their physical properties (Gilpin and Yang, 2017). Considering chemical decellularization processes, these can be further sub-divided into the use of surfactants, acids / bases and enzymatic methods. Gilpin et al, have previously described the role of each surfactant in cell membrane lysis. Solubilizing the cell membranes through acid and base techniques can result in alterations in the ECM, as well as further mechanical issues or insufficient cell removal (Gilpin and Yang, 2017). Finally, enzyme treatments can complement these techniques. Combined strategies have already proven to be effective and may represent promising strategies in the future (He et al., 2016).

Carrier-free in vivo implantation. Carrier-free approaches have shown encouraging preliminary results, with posterior corneal spontaneous adhesion assumed after anterior chamber injection of *in vitro* expanded CEnCs treated with ROCK-Inhibitors (Y-27632 at 100 µM) (Kinoshita et al., 2018). Despite these encouraging results for cell density, corneal thickness, improvement of visual acuity and persistence of cells 24 weeks post-implantation, this method is limited by protocol constraints (the patient required to bend over for 3 hours to promote cell adherence on the posterior cornea). This point raises questions concerning the clinical feasibility and reproducibility of this approach. Moreover, the use of Fetal Bovine Serum (FBS) in this procedure increases the intraocular pressure and has the risk of systemic dissemination, with consequent potential for cell proliferation, raising an issue with safety (Vázquez et al., 2016).

Cell carrier-proliferation issues and perspectives. Managing CEnCs proliferation requires a balance to be maintained between *in vitro* proliferation but then restricted *in vivo* expansion, to maintain the physical characteristics of the membrane. Under physiological conditions, the aqueous humor contains both stimulatory (bFGF) and inhibitory (TGF-beta) factors controlling CEnCs proliferation (Lu et al., 2006). Since *in vivo*, TGF-beta can inhibit the stimulatory effect of bFGF on CEnCs proliferation, it seems likely that an optimal concentration of exogenous TGF-beta may be necessary to manage

CEnCs proliferation *in vivo*. As it has already been shown that ROCK-inhibitors promote *in vivo* proliferation, it seems also likely that ROCK-stimulators could be useful in improving CEnCs outcomes (Meekins et al., 2016). Dexamethasone (DXM), a glucocorticoid, in different concentration ranges also has distinct effects on CEnCs proliferation. In bovine CEnCs, 10^{-8} to 10^{-6} M DXM enhanced Na^+/K^+ -ATPase activity with no effect on apoptosis or proliferation, while 10^{-4} to 10^{-3} M DXM increased apoptosis and necrosis, suggesting the optimal concentration still needs to be determined (Chen et al., 2006).

Similarly, oxygen levels play a role, with a level of 6% O_2 being associated with an increase in bovine CEnCs proliferation *in vitro*, while 20% O_2 significantly decrease bovine CEnC proliferation (Zagörski et al., 1989). Moreover, 0.5% O_2 (hypoxia) has been reported to be a protective factor for mechanical stress in human CEnCs, suggesting that hypoxia could manage perioperative surgical stress outcomes (Bhadange et al., 2018).

5. Experimental models of endothelial corneal disease therapy

Compared with cell culture models, animal models offer a multi-level approach, integrating macro and micro-environmental influences, as well as systemic hormonal or immune responses, and exogenous interactions. Animal models are necessary to specifically investigate surgical transplantation or implantation of CEnCs \pm carriers, as well as to investigate issues including biodegradability, immune-tolerance, and long-term outcomes. However, the management of animal care and use for scientific purposes raises ethical considerations and regulations (Table 3).

5.1 Cat model

Cats were the first species to be used as a transplantation model for CEnCs in 1979. The successful transplantation of bovine CEnCs onto cat corneal button was described, with an absence of corneal edema but including side effects on corneal transparency, and hexagonal morphology of the CEnCs (Gospodarowicz et al., 1979). Due to the useful eye size in cats, which facilitates surgical procedures, and the similarity to human corneal endothelium (Cohen et al., 1990), almost all of the existing

therapeutic approaches (corneal button, coated lens, amniotic membranes, carrier-free implantation) have been tested on cat models. Recently, a carrier-free approach using injection into anterior chamber of cat eye was reported (Kiełbowicz et al., 2010). Briefly, cat CEnCs were cultured in the presence of 10% FBS, on collagen-coated dishes, and were then injected into one eye, with each animal being its own control. This produced significant adhesion at day 7 with functional and morphological capacities present at day 30. However, data on corneal thickness and transparency are lacking. In addition, the use of cat CEnCs cultured for two weeks on human denuded DM have also been reported and exhibited good physiological morphology. These cells were positive for ZO-1 and Na⁺/K⁺-ATPase immunostaining (Proulx et al., 2009). However, neovascularization with retro-corneal membrane (Mohay et al., 1994), initial and continuing corneal edema with partial transparency (Wencan et al., 2007), and the increasing ethical issues raise doubts about this model as a sustainable model for CEnCs transplantation.

5.2 Rabbit model

Similar to results in cat models, transplanted rabbit CEnCs have been reported to exhibit ZO-1 and Na⁺/K⁺-ATPase immunostaining and show reduced corneal edema despite the use of 15% FBS (Yoshida et al., 2017). CEnCs from a human donor, expanded *in vitro* on collagen sheets, then transplanted into the anterior chamber of rabbit eyes showed unaltered pump function 28 days post-surgery (Mimura et al., 2004b). Experimental protocols using human CEnCs extracted from non-usable grafts (human corneal endothelial rings), seeded on collagen I and IV membranes from unused bone chips, showed a complete restoration of corneal function at 6 weeks in New Zealand white rabbits (Vázquez et al., 2016). A carrier-free protocol was also performed in rabbits and showed both microscopically (adhesion to DM, tight junctions with positive ZO-1 and positive Na⁺/K⁺-ATPase immuno-staining) and clinically relevant results (restoration of corneal clarity and thickness) (Jia et al., 2018). However, because of a lack of long-term follow-up and safety issues, further studies are necessary. Although rabbits represent a low-cost model (feeding, size etc...), they do present several drawbacks including remnant proliferation *in vivo*, a shallow anterior chamber, a

less rigid cornea and higher posterior chamber pressure, making surgical transplantations in rabbits complex, often requiring simultaneous vitrectomy (Yoshida et al., 2017).

5.3 Monkey models

Monkey models have been less frequently used to test CEnCs implantation. Monkey CEnCs cultured on collagen I before being re-implanted in the anterior chamber, exhibit a long-term (4 years) improvement in corneal thickness and cell density (Koizumi et al., 2008). Similarly, monkey vascular endothelial cells seeded on allogenic monkey DM (prepared by stripping) showed an improvement in corneal transparency at 90 days (Zhu et al., 2015). However, corneal edema, neovascularization and shallow anterior chambers were significantly greater in the experimental group compared with the control group. These studies also underline that monkey CEnCs exhibit poorer proliferative capacities compared with other animal models, though they are still more proliferative than human CEnCs (Yoshida et al., 2017).

5.4 Rodent models

Although widely used in research, rat or mouse models do not represent an adequate model for investigation of corneal endothelial cell therapies. Indeed, the small eye size makes the surgical transplantation procedure difficult and non-reproducible. Despite these limitations some studies have been described. In 2004, Mimura et al., using human CEnCs, performed an experimental transplantation in a nude rat model (Mimura et al., 2004a). Human CEnCs from corneal rims were seeded onto denuded Lewis rat DM and then re-transplanted through PK. The results showed, 28 days after surgery, a decrease in corneal thickness associated with an increase in endothelial cell density *in vivo*, and with an acquisition of hexagonal morphology in the treated rats. However, the short follow-up and the use of 15% FBS in this protocol, limit the translation of these results to humans.

5.5 Pig models

Pig models have stimulated widespread interest in eye research, mainly due to the similarities of human and porcine anatomy, as well as their similar physiology and immune systems (Giraud et al., 2011). Furthermore, the large body-size in pigs permits surgical procedures that are similar to those in humans. In addition, pig endothelial cells have minimal proliferative capacity *in vivo*, similar to human endothelial cells, with a mean endothelial cell density in the cornea of around 4,000 cells/mm² (Proulx and Brunette, 2012). However, the pig eye model has not generally been used for full-thickness corneal transplantation in living animals, nor for corneal endothelial tissue engineering studies, due to their size, the care facilities required and the relatively high cost.

Lastly, it is interesting to note that all protocols discussed in each animal model have used DXM after surgical intervention, suggesting a crucial role for this glucocorticoid in CEnCs tissue engineering strategies to treat corneal endothelial diseases.

Conclusion and future perspectives

In conclusion, this review focuses on the perspective of cell therapy in endothelial cornea diseases. It is well known that, currently, no efficient therapeutic alternatives exist except corneal transplantation. This review underlines that two main *in vitro* strategies can be used: expansion of eye-native cell populations such as CEnCs or LCSCs and production and expansion of CEnCs from non-eye native cell populations (SKP, MSC, iPSC). Due to different limitations of the first strategy, the second offers real perspectives, especially iPSC. The challenge with the second strategy is to obtain a monolayer of CEnCs on a biocompatible carrier, exhibited a specific hexagonal morphology (flat cells) with a programmed cell cycle arrest. Thus, based on their corneal endothelial differentiation, iPSC cells appear to be an attractive candidate for endothelial corneal therapies. However, some issues need to be fixed to promote future clinical practice: a pure fraction of CEnCs potentially obtained by the promising SdFFF technology, an easy CEnCs injection in the patient's eye or a CEnCs culture on an implantable, bio compatible and biodegradable support adapted to transplantation (Fig. 2). To promote a further rapid transfer to clinical area, animal models are crucial, integrating macro- and

microenvironment influences, systemic hormonal or immune responses, and exogenous interactions. However, each animal model presents its advantages but also its own limitations. To treat corneal endothelial failure, grafts or tissue engineering have been the most frequently considered but other therapeutic options are also available, including phototherapeutic keratectomy, anterior stromal puncture, amniotic membrane transplantation, and conjunctival flaps. Depending on the severity of corneal edema, a combination of the above treatments may be possible. Potential new approaches in this context have also been reported and consist of treatment involving collagen cross-linking and topical Rho-associated kinase inhibitors. However, these approaches only represent symptomatic treatments, especially reducing the discomfort and pain caused by corneal edema, but without effect in improving vision. These perspectives need further investigations.

Figure

Figure 1: Cornea's layer. The cornea is composed by five distinguishable layers, three of these are cellular (epithelium, stroma and endothelium), and two acellular (Bowman's and Descemet's membranes).

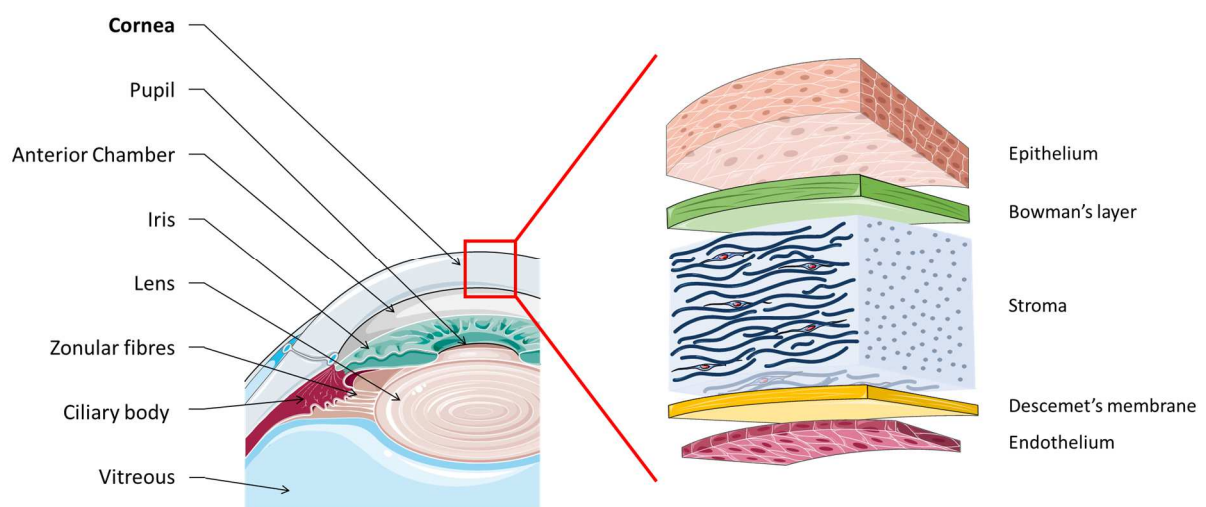
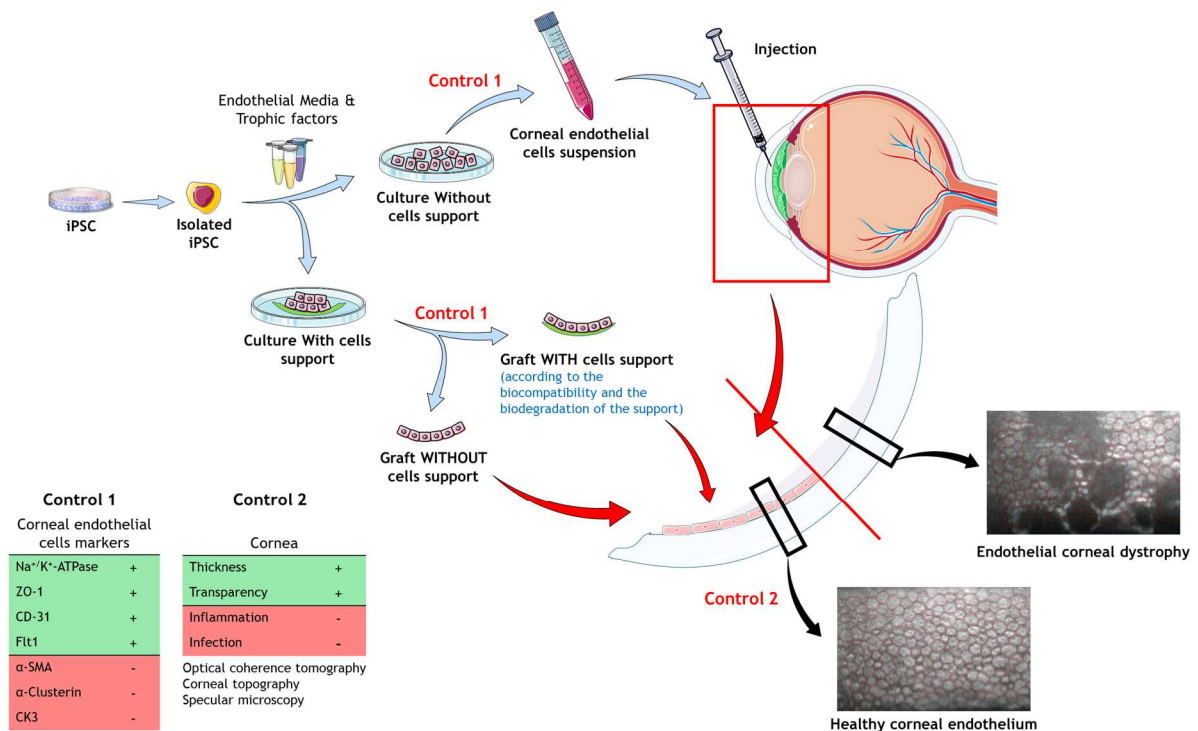


Figure 2: Evaluation of iPSC therapy strategies to treat corneal endothelium dysfunction. The first common step is to isolate iPSCs. Then, option 1: they are cultured and differentiated without support in order to have typical corneal endothelial cells (characterized by morphology and labelling) (Control 1), before injection in the anterior eye chamber; option 2: iPSCs are cultured and differentiated on supports in order to have typical corneal endothelial cells (Control 1: morphology, labelling). The new endothelium will then be grafted, with or without the support, according to its biocompatibility and biodegradation. During follow-up, several characteristics need to be investigated *in vivo* including corneal thickness, transparency, inflammation and infection, using different tools such as Optical coherence tomography, Corneal topography or Specular microscopy.



Table

Table 1. Summary of approaches of three different protocols used for the differentiation of iPSC

to CEnCs

Embryogenic-mimicking iPSC differentiation			
References	(Ali et al., 2018)	(Zhao and Afshari, 2016)	(Wagoner et al., 2018)
Origin of Cells	Human peripheral blood monocytes (PBMC)		Human fibroblasts
Coating	Matrigel coated-plates		Laminin-521-coated plates
EFSC Media		<ul style="list-style-type: none"> • DMEM/F12, N2, B27, BSA, L-Glutamax, MEM non-essential amino acids, beta-mercaptoethanol + bFGF. • At quasi-confluence, add SB431542 and LDN193189, IWP2 (6 days). 	
NCC Media	<ul style="list-style-type: none"> • mTeSR1 (4days) • 80% DMEM-F12 20% KSR, non-essential amino acids, L-glutamine, beta-mercaptoethanol and bFGF + Noggin (2 days). 	<ul style="list-style-type: none"> • low density on Matrigel + (DMEM/F12:Neurobasal 50/50, N2, B27, 2-phospho-L-acid ascorbic + CHIR99021) + passaged at 80% confluence 	<ul style="list-style-type: none"> • DMEM/F12, BSA, Trace elements A,B,C, sodium L-ascorbate, Transferrin, Heregulin β-1, LONGR3 IGF-I, bFGF) + SB431542 and CHIR99021. for 3-17 days
CEnCs	<ul style="list-style-type: none"> • 80% DMEM-F12 20% KSR supplemented with B27, PDGF-BB, DKK-2, non-essential amino acids, L-glutamine, beta-mercaptoethanol and bFGF (1 day) • Passage at day7 + 13 days with same media. 	<ul style="list-style-type: none"> • low density + coated with FNC coated mix for 24h with previous media • then at 24h new media (human endothelial-SFM, FBS, 2-phosphate acid ascorbic + SB431542 + H-1125) 	<ul style="list-style-type: none"> • NCC without supplementation + B27, PDGF-BB and DKK-2 switch daily for 30 days then 3days/week.
Time (days)	20	25	25 - 96

Table 2. Advantage and Disadvantage of support decellularization

Technics	Advantages	Disadvantages	References
Mechanical scrapping processes			
Freeze-thaw cycles method (3 Cycles: -80°C / 37°C)	<ul style="list-style-type: none"> ▪ Preservation of biochemical aspects ▪ Easy 	<ul style="list-style-type: none"> ▪ Poor decellularization (only 12% fibroblast removed) ▪ Immune rejection remain ▪ Potential contamination 	(Gilpin and Yang, 2017) (Navaratnam et al., 2015)
Supercritical carbon dioxide method	<ul style="list-style-type: none"> ▪ Maintain extracellular matrix proteins in native form ▪ Prevent the loss of physical properties 	<ul style="list-style-type: none"> ▪ Requires a long learning 	(Gilpin and Yang, 2017).
High hydrostatic pressure method	<ul style="list-style-type: none"> ▪ Partial cells membranes destruction 	<ul style="list-style-type: none"> ▪ Requires specific treatment (DNase I, glucose polymer, dextran and glycerol) ▪ Denatures the extracellular matrix proteins 	(Gilpin and Yang, 2017)
Chemical decellularization processes			
Surfactants method		<ul style="list-style-type: none"> ▪ Alterations of the extracellular matrix 	
Acid and bases method	<ul style="list-style-type: none"> ▪ Solubilizing cell membranes 	<ul style="list-style-type: none"> ▪ Alterations of the mechanical capacities 	(Gilpin and Yang, 2017).
Enzymatic method		<ul style="list-style-type: none"> ▪ Insufficient cell removing 	

Table 3. Advantages and Disadvantages of Experimental Models (CEnCs: Corneal Endothelial; Cells; FBS: Fetal Bovine Serum)

Models	Advantages	Disadvantages	References
Cat	<ul style="list-style-type: none"> ▪ No corneal edema ▪ Human-like eye sized ▪ Adapted and facilitated surgical procedures 	<ul style="list-style-type: none"> ▪ Altered transparency ▪ Altered morphology ▪ Retro-corneal neovascularization ▪ Animal ethical issues 	<ul style="list-style-type: none"> (Gospodarowicz et al., 1979) (Cohen et al., 1990) (Kielbowicz et al., 2010) (Proulx et al., 2009) (Mohay et al., 1994) (Wencan et al., 2007)
Rabbit	<ul style="list-style-type: none"> ▪ Reduced corneal edema ▪ Unaltered pump functioning <i>In vivo</i> corneal restoration 	<ul style="list-style-type: none"> ▪ Remnant proliferation <i>in vivo</i>. ▪ Surgical difficulties (shallow anterior chamber, a less rigid cornea and higher posterior chamber pressure) 	<ul style="list-style-type: none"> (Yoshida et al., 2017) (Mimura et al., 2004b) (Vázquez et al., 2016) (Jia et al., 2018)
Monkey	<ul style="list-style-type: none"> ▪ Promising results ▪ Corneal transparency 	<ul style="list-style-type: none"> ▪ Lack of insight ▪ Neovascularization + shallow anterior chamber ▪ Poorer proliferative capacities of CEnCs 	<ul style="list-style-type: none"> (Koizumi et al., 2008) (Zhu et al., 2015) (Yoshida et al., 2017)
Rodent	<ul style="list-style-type: none"> ▪ Promising preliminary results 	<ul style="list-style-type: none"> ▪ Very small sized eyes ▪ Surgical difficulties ▪ Lack of research done ▪ Short follow-up ▪ FBS used in all described protocols 	<ul style="list-style-type: none"> (Mimura et al., 2004a)
Pig	<ul style="list-style-type: none"> ▪ Human similar anatomy, immune system, physiology and proliferative capacities ▪ Human surgical procedures 	<ul style="list-style-type: none"> ▪ High-cost model ▪ Lack of study for endothelial engineering 	<ul style="list-style-type: none"> (Giraud et al., 2011) (Proulx and Brunette, 2012).

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Declaration of interests

The authors have no conflict of interest to declare.

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