Review

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Regenerative Medicine

Advances in corneal cell therapy

Corneal integrity is essential for visual function. Transplantation remains the most common treatment option for advanced corneal diseases. A global donor material shortage requires a search for alternative treatments. Different stem cell populations have been induced to express corneal cell characteristics *in vitro* and in animal models. Yet before their application to humans, scientific and ethical issues need to be solved. The *in vitro* propagation and implantation of primary corneal cells has been rapidly evolving with clinical practices of limbal epithelium transplantation and a clinical trial for endothelial cells in progress, implying cultivated ocular cells as a promising option for the future. This review reports on the latest developments in primary ocular cell and stem cell research for corneal therapy.

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Corneal biology

The human cornea is a 550-µm thick, transparent, dome-shaped structure covering the front of the eye. It serves three fundamental functions: first, mechanical and chemical barrier protecting the inner eye tissues; second, a high degree of transparency for light transmission, and third, light refraction (providing two-thirds of the eye's focusing power). The clarity is maintained by first, anatomical features – keratocytes biosynthesizing crystallins and organizing regularly arranged collagen lamellae and second, physiological characteristics – relative avascularity and corneal dehydration regulated by corneal endothelial cells and barrier function of the epithelium and endothelium to control fluid passage (Figure 1) [1].

The nonkeratinized squamous corneal epithelium is continuously regenerated by limbal stem cells (LSCs) that reside in the palisades of Vogt of the peripheral cornea. Damage to this region can lead to irreversible limbal stem cell deficiency (LSCD), resulting in impaired regeneration of corneal epithelial cells (CEpCs) and keratopathy [2,3]. The stroma located beneath the epithelium comprises about 90% of corneal thickness. Its biomechanical and transparent characteristics are due to the unique arrangement of collagen lamellae and extracellular matrix (ECM) produced and maintained by corneal stromal keratocytes (CSKs) [1]. Corneal stromal stem cells (CSSCs) have been identified within the limbal stroma [4]. Infection or injury can cause formation of stromal scars and opacities leading to vision loss [5]. The single-layered corneal endothelium with its functional pumping activity regulates the stromal hydration state to maintain corneal transparency [6]. Even though progenitors are suspected to populate in the posterior limbal area [7], human corneal endothelial cells (hCECs) are relatively nonproliferative *in vivo*. Cell loss occurs due to aging, trauma or iatrogenic factors, causing corneal edema and deterioration of vision. Corneal endothelial disorders currently represent the

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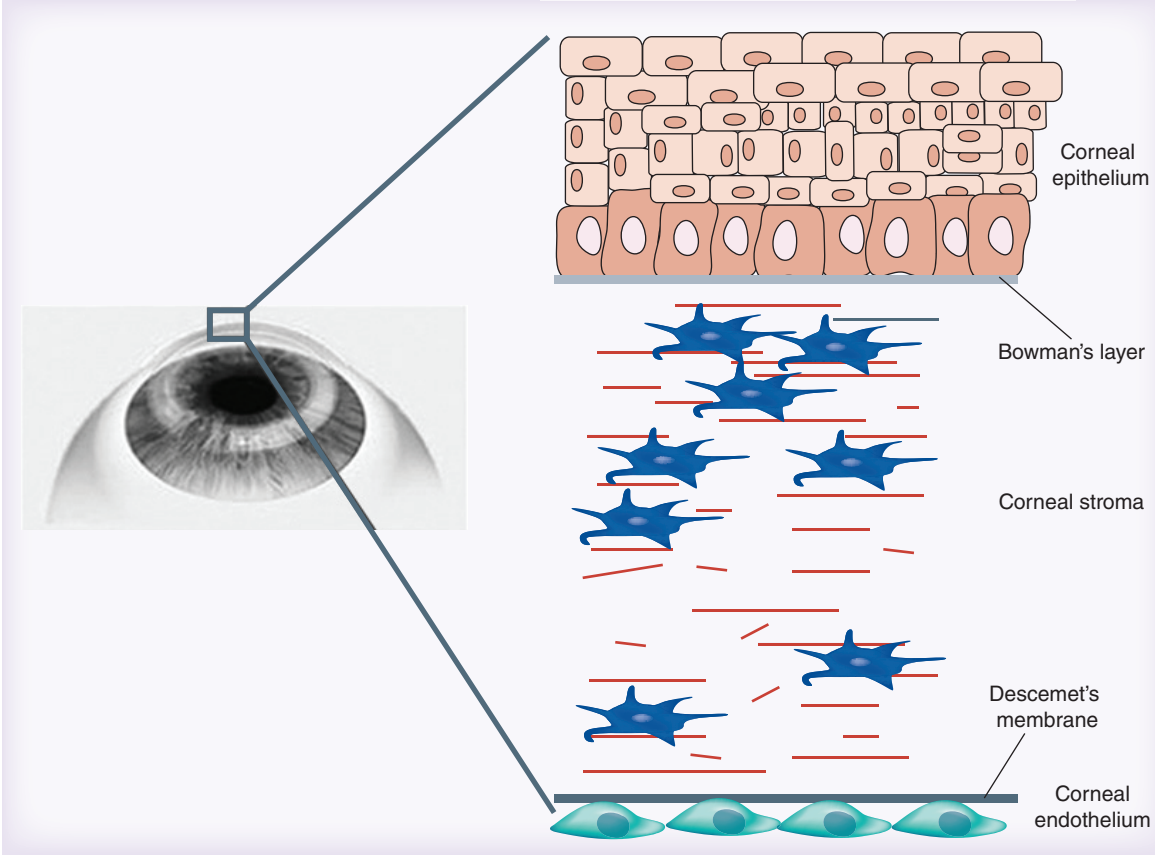

Figure 1. The human cornea consisting of five known layers, three cellular (epithelium, stroma and endothelium) and two interface lamellae (Bowman layer and Descemet membrane).

most common indication for corneal transplantation in developed countries [8].

So far corneal transplantation is the preferred treatment option for advanced stages of stromal and endothelial disorders. Despite tremendous advancements to the surgical techniques over the past decade, there are still many factors that hinder its long-term success including global donor material shortage, limited graft survival, allogeneic graft rejection, use of immunosuppressants, high surgical costs, prolonged postsurgery management and a need of high-level surgical expertise to perform the procedure [8,9].

Although the total number of donors and eye globes/corneas donated has been increasing in recent years (a rise of 5.2% in 2013 compared with 2012, data from Eye Bank Association of America), the global population is expected to increase to 113% by 2030 and life expectancy will rise at a 0.07% annual rate (data from Department of Economic and Social Affairs, Population Division, United Nations). Hence, this will propagate the worldwide issue of donor material shortage. Even in countries with a well-developed eye banking system, for example, the USA and western Europe, many potential donor tissues are eliminated due to the positive testing for transmissible viruses (like hepatitis B and C carriers increased by 275% and 241% from 2006 to 2011, Figure 2) [10]. Other factors, such as long-term medication history and religious constraints, also reduce the donor pool with a prediction of an increase of unsuitable tissue to 237% by 2030. Hence, alternative solutions, such as regenerative therapy using cultivated cells, should be explored.

Corneal cell therapy

Regenerative cell therapy could bypass many complications of conventional corneal transplantation and has gained increasing interest in recent years. The human cornea is an ideal organ for cell therapy, as it is avascular and immune-privileged, hence transplanted cells are not as likely to be rejected as in other locations. Developments in the field of stem cell (SC) engineering, particularly with the use of autologous tissue, have generated significant interest among ophthalmologists. Pluripotent embryonic SCs (ESCs) are self-renewing and represent a potentially infinite source that can differentiate

into virtually any cell type. However, differentiated cell purity, identity and the risk of teratoma formation limit the implementation from experimental results toward a clinical reality [11,12]. Multipotent mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs) are derived from adult tissue. Their applications avoid the controversial ethical issues, and the need for aggressive post-transplantation control for immune-mediated rejections, especially when these cells can be obtained from autologous sources. However, the widespread utilization of iPSCs is limited by its low reprogramming efficacy, the lack of standard protocols to derive corneal cells, potential risks of oncogenic transformation and the problematic epigenetic memory [13]. More work is required to optimize the derivation and differentiation procedures, before they can be safely and reliably employed in corneal tissue engineering.

The harvest, expansion and reimplantation of primary human corneal cells, on the other hand, have made substantial progress in recent years, offering the prospects of targeted cell therapy, which we will review in this article.

Epithelial cell therapy

The limbus with its rich vasculature and papillary structure (palisades of Vogt) functions as a niche for LSCs and regulates their survival and self-renewal as well as protecting them. Following the asymmetric division of SCs, daughter cells migrate out from the niche to become transit-amplifying cells, which proliferate and differentiate into progeny of CEpCs [2]. Damage to the limbus may reduce or even destroy this stem cell population, resulting in defective cell renewal and epithelium regeneration. LSCD can be congenital (e.g., in aniridia) or acquired (e.g., in cases of Stevens–Johnson syndrome, ocular cicatricial pemphigoid, contact lens-induced keratopathy, acid and alkali burn injuries) [3]. Patients normally present with corneal neovascularization, chronic inflammation, persistent and recurrent epithelial defects, and conjunctivalization, resulting in decreased visual acuity, increased tearing, recurrent pain, photophobia, blepharospasm and symblepharon [3].

LSC transplantation

Limbal autografting

The treatment depends on whether the patient has unilateral or bilateral disease and on the degree of limbal damage. For partial LSCD, in which the deficiency involves a few sectors of limbus, good clinical results can be achieved with mechanical debridement of the encroaching conjunctiva, in conjunction

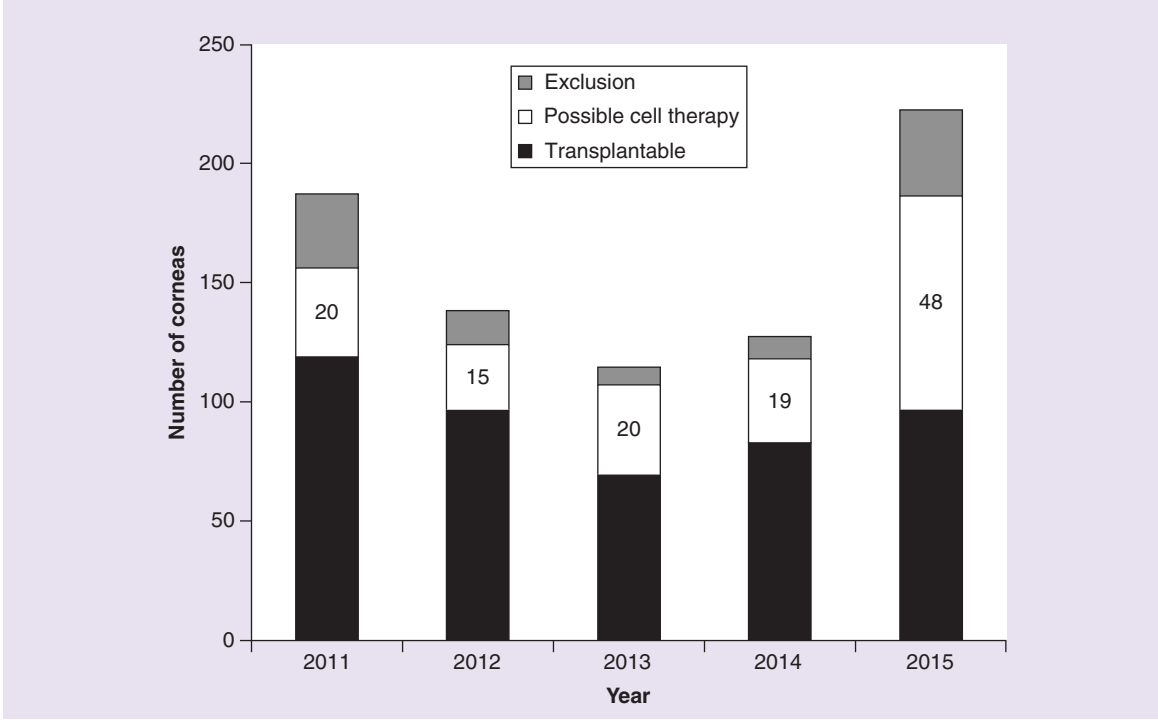

Figure 2. Corneas processed by the Singapore Eye Bank in years 2011–2015. Transplantable corneas had no limitations, excluded transplants (positive serology, contraindications, contamination, among others) had to be discarded, possible cell therapy corneas (percentage of all corneas displayed) had low endothelial cell counts, scaring, prior refractive surgery, among others, which prevented them from being used for transplantation. However, they could represent a future source of corneal cells for cell therapy.

with the application of human amniotic membrane (hAM) [2,14]. In cases of substantial unilateral LSCD, limbal autografting can be performed. This can be achieved by three methods: conjunctival limbal autografting (CLAU), cultivated limbal epithelium transplantation (CLET) and the recent adaptation of simple limbal epithelial transplantation (SLET). The transplantation of a CLAU (Figure 3A) from the healthy eye onto the injured eye was first described by Kenyon and Tseng in 1989 [15]. Success rates up to 82% have been reported for this therapeutic procedure [2,3,16].

Cultivated limbal epithelium transplantation

CLAU itself entails a risk of LSCD for the donor eye, as well as minor complications, for example, discomfort, chronic inflammation, scarring and infection [3]. Hence, efforts have been made to minimize the size of autologous limbal graft. In 1997, Pellegrini *et al.* cultivated and expanded human LSCs *ex vivo* and successfully transplanted the cell sheets onto the corneal surface of two LSCD patients [17]. Favorable results using this method were also reported by Tsai *et al.* and Rama *et al.* [18,19]. The number of transplanted LSCs expressing ΔNp63α was found to be important for

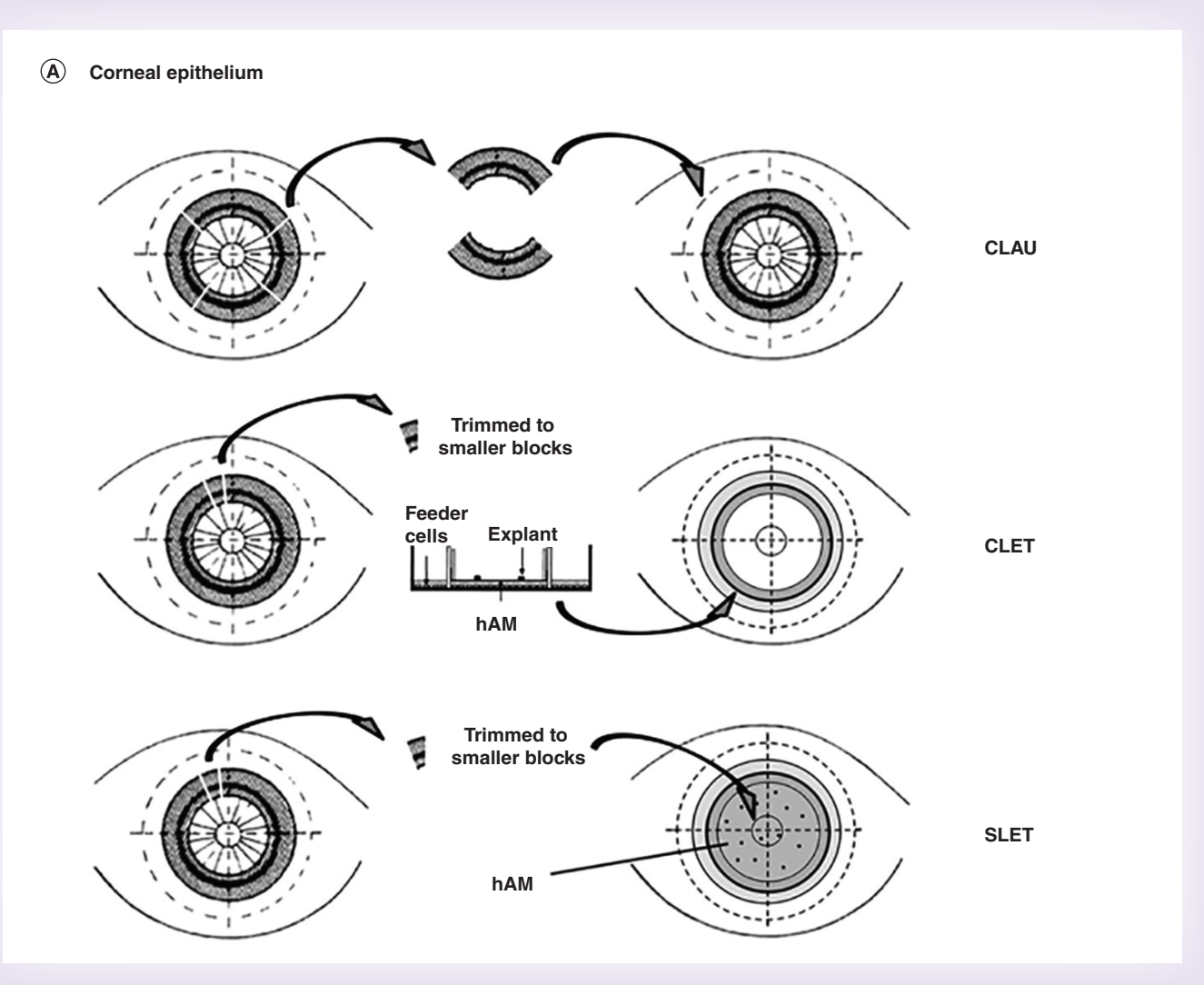

Figure 3. Corneal cell therapy. (A) Corneal epithelium can be replaced by the clinically established techniques of CLAU, CLET or SLET. **(B)** Corneal opacifications have experimentally been treated by intrastromal injections. In case of more advanced stromal damage, tissue can be replaced by stacking substrates and cells in a sandwich method, by colonizing a substrate with cells *ex vivo* followed by transplantation or by transplanting a cell-free substrate that is subsequently invaded by host cells. **(C)** Endothelial cell therapy can theoretically be achieved by direct cell injection into the anterior chamber and prone positioning of the patient to facilitate cell attachment to Descemet membrane or by expanding human corneal endothelial cells *ex vivo* on a TE-DSEK lamella, which is then implanted.
CLAU: Conjunctivolimbal autograft; CLET: Cultivated limbal epithelium transplantation; TE-DSEK: Tissue-engineered Descemet Stripping Endothelial Keratoplasty; hAM: Human amniotic membrane; SLET: Simple limbal epithelium transplantation.

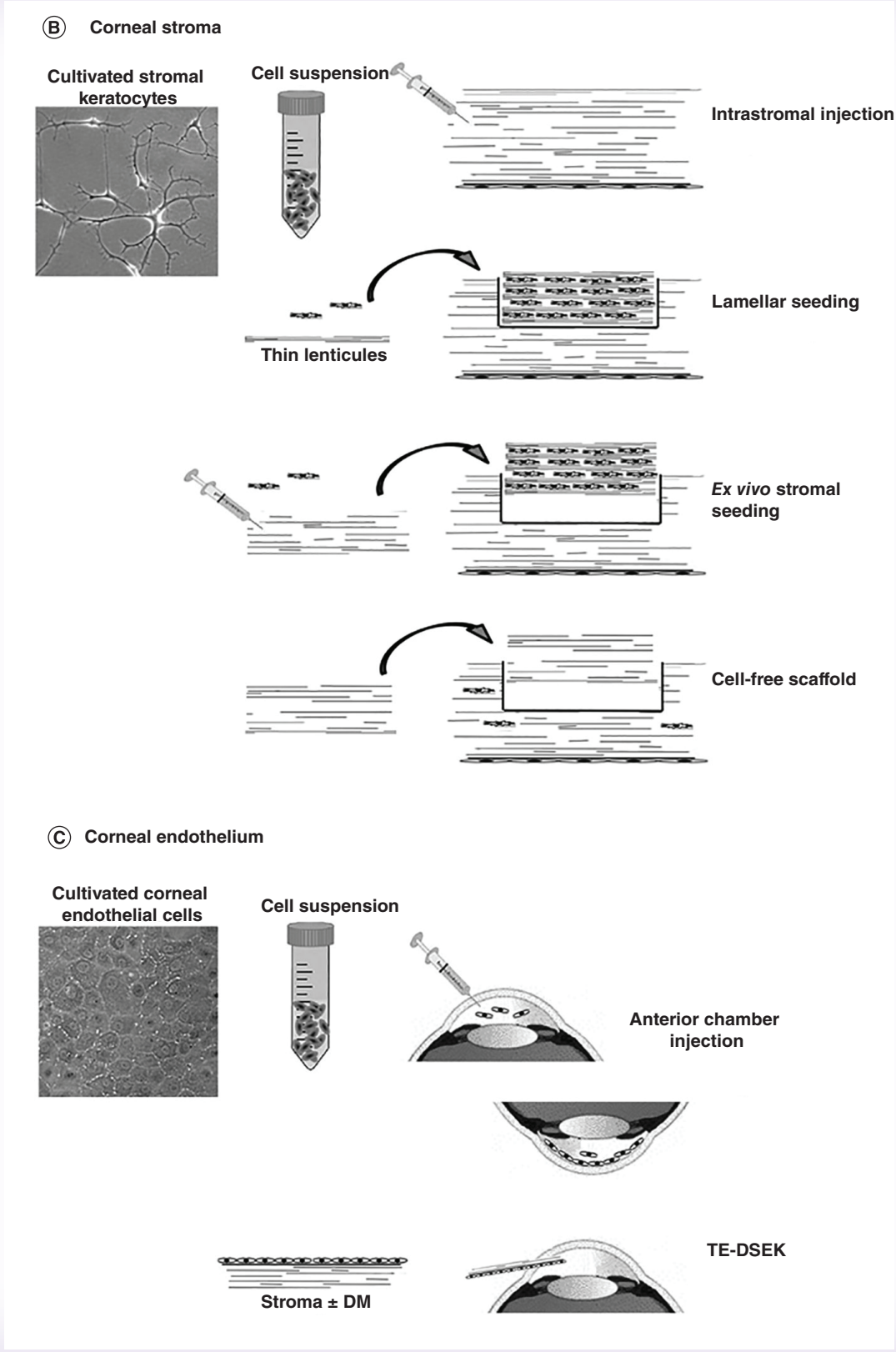

Figure 3. Corneal cell therapy (cont.).

long-term graft survival. Different culture protocols have been reported, including 'explant' techniques by directly placing limbal biopsies on a substrate and 'cell suspension' cultures using feeder cell layers. Both achieve favorable outcomes of CLET [2].

The smaller size of healthy limbal biopsy (about one-clock hour, Figure 3A) needed for CLET also allows repeated autologous transplantation [20]. The success rates (i.e., no superficial corneal vascularization, conjunctivalization or repeat epithelial breakdown) vary between 45 and 100%, depending on the degree of LSCD and other co-morbidities [2,19]. One of the major drawbacks for CLET is its high treatment cost and need for clean-room facilities, trained staff and good manufacturing practice (GMP)-qualified culture reagents, which restrict the procedure to be performed in a few specialized centers worldwide [2].

Much effort has been spent on searching for an ideally biocompatible, mechanically stable, optically transparent substrate that allows efficient cell adhesion, migration and proliferation for the *ex vivo* expansion of LSCs and subsequent delivery [14,21,22]. To date, hAM is the most widely used biological matrix, due to its ability to promote epithelialization with its inherent growth factor content, its low immunogenicity, antimicrobial, antiviral, antifibrotic and antiangiogenic properties [14]. In addition, hAM can easily be trimmed to conform to the desired ocular surface area, and it can be efficiently anchored by sutures or fibrin glue. However, even though unlikely, the possibility of disease transmission may occur but can be reduced by stringent screening of transmissible diseases in donors. Also variable quality of material and reduced transparency have led to the investigation of biological and synthetic alternatives:

- Collagen, being a major component of the corneal ECM and basement membrane, is naturally biocompatible, has low immunogenicity and production cost. Collagen substrates have been shown to promote CEpC growth *in vitro* and in animal models [22]. However, the high water content reduces the stability of collagen hydrogels, which can be improved by mechanical compression or chemical cross-linking [21]. Nevertheless cross-linkers can be cytotoxic and reduce cell viability, long-term epithelial stability, as well as matrix remodeling by the transplanted cells [21,22]. Recombinant collagens offer high purity and further reduce immunological concerns, yet their production costs are substantially increased [22]. Collagen vitrigel membranes have superior optical and mechanical properties, but require long dehydration times [21,23];
- Fibrin sealant has proven to be a suitable substrate for LSC expansion in the treatment of more than 113 LSCD patients [19,24]. However, it may induce limbal cell differentiation to express CK3, a differentiated epithelial cell marker [25];
- LSCs were demonstrated to form epithelial sheets on temperature-responsive surfaces; these substrates swell or degrade due to changes in temperature but the released cell sheets lack mechanical stability [26];
- Silk fibroin, a protein isolated from the cocoon of the silkworm *Bombyx mori*, did not induce any immunogenic response after implantation *in vivo* and promoted CEpC growth [27]. However, high production costs might limit its application;
- Other biological materials that have undergone *in vitro* studies are lens capsule and keratin. Though LSCs have been successfully cultured on human anterior lens capsule, availability and fragility are major limitations on further research and application [28]. Keratin films have much higher light transmission capacity when compared with hAM; however, suture placement on these films is difficult with a higher rate of suture loosening, resulting in poor anchorage to the ocular surface [29].
- A variety of synthetic materials have also been investigated. A clinical trial reported the successful cultivation and application of LSCs on siloxane-hydrogels (contact lenses) [30]. Polycaprolactone substrate has been shown to facilitate effective cell attachment in animal studies [31]. To date results for gelatin-chitosan, the US FDA approved poly(lactide-co-glycolide) (PLGA), hydroxymethylacrylate and polymethacrylate carriers are limited to *in vitro* studies [22]. Further characterizations and *in vivo* investigations are necessary to evaluate their potential.

In order to minimize the cost and complexity of CLET, SLET was developed; a one-step surgical procedure combining the placement of healthy limbal tissue fragments on hAM, which was directly anchored on the recipient's cornea (Figure 3A) [32]. It adapts the small biopsy size as in CLET, while cell growth is taking place *in vivo*, instead of in a laboratory. This tremendously reduces the culture preparation period, the need of culture expertise and GMP facilities, resulting in reduced costs and shorter treatment times. Two clinical trials of up to 11-month follow-up have shown the restoration of a stable epithelial surface and considerable improvement in visual acuity of all patients with no reports of complications [32,33]. However, the long-

term efficacy and treatment outcome are yet to be evaluated, as this method does not allow the quantification and enrichment of (ΔNp63α-positive) LSCs.

Bilateral LSCD

In cases of bilateral LSCD, where autologous LSC transplantation is impeded, other autologous nonocular or allogenic sources, such as living relatives or cadaveric donors, are required. This decreases the success rates and patients are often burdened with long-term immunosuppression [2].

Other cell sources for ocular surface reconstruction

- In LSCD cases, the conjunctival integrity is also affected, leading to a loss of essential goblet cells and dry eye pathology [34]. Conjunctival epithelial cells show similarities to CEpCs and several groups have successfully reconstructed the ocular surface by transplanting cultivated conjunctival epithelial cells [35];
- Transplantation of cultivated oral mucosal epithelium was the first nonocular surface epithelium used for LSCD treatment [2]. Oral mucosa is similar to corneal epithelium. The stratified squamous epithelium matures without undergoing keratinization. It lacks hair follicles and sweat glands and cells regenerate rapidly. The tissue can easily be obtained from the gingiva, making it an alternative autologous cell source for bilateral LSCD. However, oral mucosa epithelium varies in its stratification and the number of cell layers, which can lead to uneven surface morphology after transplantation and suboptimal vision [2]. Unlike CEpCs, oral mucosa epithelia do not express anti-angiogenic factors, such as soluble FLT1, TIMP3 and TSP1 [36] and the majority of cases develop recurrent epithelial defects and corneal neovascularization [3]. In a large retrospective study, cultivated oral mucosal epithelium achieved improvement in vision in 48% of patients, which was maintained with median follow-up of 28.7 months [2];
- Clinical trials have been conducted using nasal turbinate to treat LSCD. While the transplantation of intraepithelial goblet cells in nasal mucosa improved and stabilized the tear film, fornix reconstruction succeeded only in 9 out of 17 patients after 6–31 months follow-up [37];
- Dental pulp stem cells (DPSCs) express markers in common with LSCs, such as ABCG2, integrin β1, vimentin, connexin43 and CK3/12 [38]. Transplantation of a tissue-engineered cell sheet was shown to reconstruct rabbit corneas of mild chemical burns. However, in severely injured animals, the reconstructed epithelium consisted of unnatural flattened cells [39];
- Human ESCs exhibited a corneal epithelial-like phenotype (expressing ΔNp63α and CK3/12) when cultured in limbal fibroblast-conditioned medium [40];
- iPSCs reprogrammed from dermal fibroblasts could generate CEpCs [13]. However, the efficacy of these cells is yet to be shown in animal models;
- Murine hair follicle bulge-derived stem cells were chemically induced to a CEpC phenotype expressing CK12 and showed 80% repopulation efficiency of the corneal surface in a mechanical mouse LSCD model [41];
- Adult MSCs are proliferative and multipotent stem cells that can differentiate into cells of various lineages. They can be harvested from autologous sources, such as bone marrow, adipose tissue and also from allogenic sources, for example, umbilical cord linings [42]. Changes in cell phenotypes from mesenchymal to epithelial state, defined as mesenchymal–epithelial transition (MET), can be manipulated by regulating various signaling pathways. Human bone marrow MSCs on hAM cultured using limbal fibroblast-conditioned medium were differentiated into corneal epithelial lineage, improving corneal healing in a rat alkali burn model [42]. However, some groups reported only minor improvements or no positive effect at all in LSCD animal models and feeder cells/conditioned medium impede the implementation of reliable protocols [3]. Our group has developed a MET protocol using a combination of small molecules inhibiting TGF-β and GSK3 signaling pathways and differentiated human adipose-derived MSCs to corneal epithelial-like cells [43]. The *in vivo* application to a rat alkali burn model greatly improved corneal clarity with minimal neovascularization and the reconstructed corneal epithelium expressed corneal epithelial markers. This suggests MET cells derived from adult MSCs as a potential source for corneal surface reconstruction.

Stromal cell therapy

The corneal stroma is composed of collagen fibrils in the form of lamellae running orthogonally to each other. Both CSKs and CECs are derived from the cranial neural crest via the intermediate periocular mes-

enchyme [44]. Adult CSKs are mostly quiescent and sparsely populated in between collagen lamellae with intercellular connection via extended dendrites. They produce collagens and keratan sulfate (KS) proteoglycans (lumican, keratocan and mimecan) for ECM assembly, and enzymes (such as collagenases) for ECM turnover and stromal modeling. These activities regulate collagen fibril growth and alignment, which are essential for corneal strength and transparency [45]. Trauma, infection, immunological disorders, inherited diseases and degeneration and/or induced injuries can lead to CSK death or transformation to stromal fibroblasts, resulting in corneal opacities and reduced visual acuity. Over 10 million people worldwide are affected by corneal opacities. Surgical removal can restore their eyesight [5]. Even though development of eye bank facilities and refinement of surgical procedures for penetrating and lamellar corneal transplantation have considerably improved our ability to treat corneal blindness in recent years, widespread accessibility to modern day surgery is still limited worldwide, often due to continued donor material shortage and lack of surgical expertise [8], hence targeted cell therapy may represent a desirable alternative.

Ex vivo CSK cultivation

Great challenges are presented for the *ex vivo* cultivation of CSKs. In the presence of serum, quiescent CSKs re-enter into the cell cycle and proliferate, but they fail to maintain a keratocyte phenotype and transform into stromal fibroblasts, including: first, loss of dendritic shape while acquiring a bipolar morphology and stress fiber formation; second, loss of *CSK* gene profile and activation of α5-integrin and αSMA and third, halted production of KS-containing proteoglycans [46,47]. Using soluble human amnion stromal extract, ROCK inhibitor (Y27632), IGF1 and low serum content, 'activated keratocyte' populations can be propagated *ex vivo* [47]. When cells returned to serum-free conditions, they regained CSK marker expression (including keratocan, lumican, ALDH3A1) and displayed negligible fibroblastic phenotypes. Although there was a variability in cell yield, due to donor-to-donor variation (constraining factors include age of donor, cause of donor death, corneal preservation time and condition), this culture protocol can propagate CSKs from one stroma to be sufficient for the engineering of approximately five full thickness stromata. This would provide a therapeutic potential for multiple patients. Further tests in animal models will ascertain the potential of these cells.

Other cell sources

The discovery of ABCG2-expressing CSSCs in the limbal stroma, which demonstrated clonal growth *in vitro* and differentiation into cells expressing typical keratocyte markers (keratocan, ALDH3A1 and KS), has stimulated further research on stromal regeneration [4]. CSSCs in pellet culture under serum-free condition-expressed keratocan, KS, collagen I, V and VI and organized orthogonally oriented collagen fibrils in multilayered lamellae strongly mimicking human corneal stromal tissue [48]. Direct intrastromal injections of CSSCs could remove stromal opacities in *lumican* knockout mice [49]. However, CSSC differentiation may derive other cell types such as fibroblasts, indicating possible contamination problems.

Other cell sources have been shown to differentiate into keratocytes. Human ESCs, via neural crest induction and enrichment, could generate to keratocan-expressing cells *in vitro*. Nevertheless, cell heterogeneity and tumorigenecity may pose a problem for translational use [50]. IPSCs have been generated from stromal keratocytes, yet redifferentiation to functional CSKs has not been described [51]. MSCs from bone marrow, adipose tissue and umbilical cord have all been used for *in vitro* stromal reconstruction. Intrastromal injection of these cells to *lumican* null mice-derived cells with keratocyte phenotype, resulting in improved corneal transparency [52]. MSCs are known to suppress immune reactions, reduce corneal neovascularization and possibly graft rejections. However, the presence of non-CSK cell types, such as fibroblasts, once again poses an issue in translational use. Recently, human DPSCs could differentiate into CSKs *ex vivo* in the presence of bFGF, TGF-β3 and ascorbate-2-phosphate [53]. Intrastromal injection to a mouse model resulted in clear corneas with the production of human collagen I and keratocan. This represents a potential use of nonocular adult stem cells for ocular cell therapy.

Stromal therapy

Cell injection

To date, the *ex vivo* expansion of adult hCSKs from donors unable to be used for corneal transplantation due to limitations seems to be the most cost-effective approach for stromal cell therapy. In 2015, 48% of 222 donor corneas at the Singapore Eye Bank were disqualified to be used for penetrating or endothelial keratoplasty, due to low endothelial cell counts, scars, among others. (Figure 2). They represent a potential source for CSK isolation, cultivation to greater numbers and intrastromal injection to localized regions, in for instance scar treatment (Figure 3B) [49].

Tissue engineering

While CSK injection is a possible cell therapy approach for mild-to-moderate corneal scars and defects, severe stromal diseases currently still require total stromal

replacement by penetrating or deep anterior lamellar keratoplasty [8]. In the future stromal tissue could be replaced by cell seeding on decellularized lenticules, followed by stacking to obtain a sandwich configuration [54], or transplantation of matrices with or without *ex vivo* cell seeding, followed by a slow process of cell migration and ECM reorganization (Figure 3B) [55,56].

Different substrates have been tested for stromal tissue engineering. Similar to epithelial cell therapy, collagenous materials with cross-linking or mechanical compression have been shown to improve construct stability with limitations in cell viability and matrix remodeling [21,56]. Recombinant products can further reduce immunological reactions but are currently expensive. In a recent clinical study, ten keratoconus patients undergoing anterior lamellar keratoplasty with cross-linked recombinant collagen grafts vision improved in five patients, while in one case, the construct was rejected during the 4-year follow-up. Reinnervation and stromal remodeling by migrating CSKs was observed. However, subepithelial fibrosis and implant thinning occurred in 70% of transplanted patients, which could be due to high suture tension on this relatively soft collagen material leading to surface irregularities and delayed epithelialization [56,57]. Collagen vitrigels are composed of a high proportion of water, which leaves them intrinsically weak unless modified with chemical cross-linking or blended with other polymers to create collagen composites, limiting direct seeding of cells within the scaffold. Nevertheless, they were shown to promote dendritic branch density, cell length and expression of ALDH and keratocan of CSKs *in vitro* [23]. Other substances are being tested as alternative stromal biomaterials, including gelatin, chondroitin sulfate [58] and PLGA [59].

Most tissue-engineered constructs have the common disadvantages of insufficient tensile strength, the failure to mimic native surface curvature and stromal architecture, making them unable to achieve high optical transparency. This could be resolved by decellularized corneas (DCs) from animal and human origins, as they retain the prevailing 3D ECM structure, biocompatibility, biomechanics and transparency [55]. While complete removal of cell remnants is crucial to reduce immunogenicity, the preserved ECM ultrastructure allows an efficient recellularization and high biocompatibility. So far, there is no standard protocol to decellularize corneal stroma. Different protocols on whole cornea or thin stromal lenticules yield variable efficiencies of cell removal [55]. Reimplanted DCs have been tested in animal studies, however, the results are limited by the xenogenic origin and lack of disease model to reveal the efficiency in stromal reconstruction. Recent animal studies of anterior lamellar grafting showed variations in re-epithelialization and stromal cell infiltration [55]. A clinical trial using porcine DCs for corneal repair in humans (ClinicalTrials.gov NCT01443559) has been suspended and no results were published. Nevertheless, a randomized trial comparing the implantation of fresh human corneal stroma and acellular cryopreserved stroma for deep anterior lamellar keratoplasty in high-risk patients showed significantly less rejections over 2 years in the acellular stroma group [60], indicating DCs as a promising treatment option.

Endothelial cell therapy

The monolayer of hexagonal CECs with its active Na^{2+}/K^{+} transporter function regulates the corneal hydration homeostasis ('pump–leak' hypothesis) and optimizes interlamellar spacing of collagen fibrils, resulting in corneal clarity [6]. There is an inverse relationship between age and corneal endothelial cell density [61]. Usually, the average reserve of hCECs is sufficient to maintain the critical barrier and pump function for a person's lifetime. In cases of accelerated or acute endothelial cell loss and when endothelial cell density falls below a threshold range of 500 to 1000 cells/mm^2, decompensation of the corneal endothelium and inability to efficiently pump fluid out of the stroma will result in stromal edema manifesting as corneal clouding and loss of visual acuity [6]. To date, the only option to restore vision due to endothelial cell failure is to transplant healthy, functional donor endothelium.

Corneal endothelial dysfunction remains the most frequent indication for corneal transplantation [8], making it a prime target for cell therapy. Selective endothelial replacement surgery was first described by Melles in 1998. Since then, extensive improvements in technique have given rise to Descemet Stripping Automated Endothelial Keratoplasty and Descemet Membrane Endothelial Keratoplasty with substantially improved visual outcomes [8]. However, these procedures still rely on allogeneic tissue with one-donor cornea used for one endothelial keratoplasty procedure.

Expansion of hCECs *in vitro*

hCECs were thought to be incapable of cellular division, due to their G1 arrest by contact-dependent inhibition and TGF-β2 [62]. In 1979, hCECs were first reported to undergo mitosis given the appropriate milieu [63]. Since then, various protocols, media and additives were described for hCEC propagation [61,64,65]. Nevertheless, challenges, such as restricted proliferative ability, donor-to-donor variability, cell senescence, endothelial–mesenchymal transition (EMT), the need to adapt xeno-free protocols and the mode of delivery to recipient endothelium, remain.

The supplementation of ROCK inhibitors to hCEC culture promoted functional characteristics such as cell proliferation and adherence to substrates [66–68]. Similar effects were identified in animal models [67]. The possible molecular mechanisms include promoted degradation of p27 to stimulate cell proliferation and cyclin D expression via PI 3-kinase signaling [69].

Cultured hCECs can exhibit substantial variability in proliferative and phenotypic characteristics related to donor age, background diseases, predeath drug use and graft storage conditions [70,71]. The greater proliferative potential from younger donors was explained by the process of replicative senescence in older cells [72]. Interdonor variations must be taken into account when comparing hCEC populations from multiple donors. Successful cultivation of hCECs *in vitro* represents a compromise between the intended stimulation of proliferation and the undesired induction of EMT. Several strategies have been employed to suppress EMT, for example, a dual media expansion protocol [65], TGF-β blockage, supplementation of growth media with L-ascorbate 2-phosphate, siRNA-blocking p120 activity and the inhibition of matrix metalloproteinase activity [61,73].

Endothelial therapy

Cell injection

Once hCECs are successfully propagated, they need to be delivered to the host's posterior corneal surface (Figure 3C). Endothelial monolayers cultured on stimuli-responsive polymer surfaces were too fragile for clinical use [74]. Intracameral injection of hCECs with subsequent prone posturing is an attractive approach, but there are concerns whether this technique can deliver a sufficiently high cell number in a consistent manner to the posterior surface of the cornea. Cell attachment has been facilitated by the use of ferromagnetic induction [75] or ROCK inhibitors [76]. A clinical trial evaluating this delivering technique has been initiated in Japan in 2013 (Registration Number: UMIN000012534). The study is currently on-going and results are yet to be published.

Tissue engineering

In an alternative delivery approach, hCECs are seeded on biological, for example, gelatin, collagen I gels (vitrigel), animal or human DC [76–80] or synthetic carriers, for example, chitosan, PLLA and PLGA [81,82]. These tissue-engineered DSEK lamellae have been successfully transplanted onto DM-stripped recipient corneal stromal beds in animal models (Figure 3C) [76,79]. Synthetic polymers have the benefit of high purity with known chemical composition, structure, physical properties and degradation times. However, some components may induce inflammatory reactions [82]. Biological carriers, in particular DC lamellae, advantageously represent the natural substrate for hCECs. However, they may transfer infections and xenografts can be rejected, especially in cases of insufficient decellularization. Also the use of human material does not reduce the dependency on donor tissue. Future investigations have to determine the optimal material.

Corneal endothelial stem cells, regeneration *in vivo*

A circumferential and discontinuous line of cells with unusual ultrastructural characteristics along Schwalbe's line was initially described in 1982 in monkeys [83]. Subsequent anatomical studies described progenitor cell populations at a transitional zone from the periphery of endothelium and Schwalbe's line to the anterior portion of the trabecular meshwork, referred to as posterior limbus. They could generate both endothelial and trabecular cells [7]. In addition, hCEC regeneration from the posterior limbus was evidenced by Bednarz *et al.*, showing mitogenic activity only in hCECs from the periphery but not from the central cornea [84]. hCECs from the peripheral cornea had shorter doubling times than those from the central cornea [85]. Positive telomerase activity was also detected in peripheral and intermediate sections but not in central endothelial tissue [86]. The identification of SC markers, for example, nestin, LGR5 and alkaline phosphatase, in the posterior limbus [87] supports that these cells may possess regenerative capability.

Interestingly, in patients with Fuchs endothelial dystrophy, CECs may migrate and/or proliferate over bare recipient corneal stroma leading to corneal clearance and visual rehabilitation after just central denuding of the Descemet membrane, making an endothelial transplantation unnecessary [88,89]. The term 'DMET,' or Descemet Membrane Endothelial Transfer, was thus instituted to describe such 'failed' endothelial keratoplasties, which nonetheless demonstrated relative anatomical and clinical 'successes'. Descemet Membrane Endothelial Transfer was more likely to result in corneal clearance among subjects with Fuchs dystrophy, in contrast to those with bullous keratopathy [88]. It has also been shown in a recent Phase I clinical study that Fuchs endothelial dystrophy can be treated by topical Y27632 following cryodestruction of the diseased endothelial layer [90].

Other cell sources for corneal endothelium engineering

Although there were reports of CEC generation from human ESCs [91], ethical and scientific questions, such

as low efficiency of conversion (7.7%), as well as the risk of tumorigenesis are likely to limit their clinical application [11,12]. Multipotent SCs from adult corneal stroma have been shown to derive functional CECs [92]. Skin-derived precursor cells of neural crest origin have also been differentiated to functional CECs in the presence of retinoic acid and upregulated Wnt/β-catenin signaling [93]. Transplanted CECs differentiated from monkey iPSCs to rabbit eyes have proven capable of regulating stromal hydration [94].

As CECs are developmentally derived from the cranial neural crest via the intermediate periocular mesenchyme [44], it is theoretically possible to generate CECs from PDLSCs and DPSCs by manipulating key developmental signaling, such as TGF-β and retinoic acid signaling and the induction of transcription factors (PITX2 and FOXC1) [95]. This requires a better understanding of these pathways in the context of CEC development and the temporal involvement of various signaling.

GMP-compliant cell engineering

The use of targeted corneal cell therapies will only be clinically feasible, when cells can be generated under large-scale culture, in compliance to GMP regulations and guidelines of the local authorities. This will inevitably require specialized tissue-engineering facilities, significant manpower and financial costs. Most reported protocols still rely on animal-derived research-grade products at multiple stages, in particular fetal bovine serum or cholera toxin is difficult to replace [64,65,96]. Hence, potential risks of xenogenic contamination and transfer of animal-borne infectious pathogens could limit cell therapy applications [96]. Some research groups have recently revised protocols to avoid animal-derived products in cell culture and use autologous serum and human recombinant growth factors as culture supplements [97–99]. It has also been shown that the use of clinical-grade 3T3-J2 feeder cells is safe and does not lead to cell contamination [100]. The future will be to develop xeno-free protocols for all prospective corneal cell therapies.

Executive summary

Corneal disease

- Corneal functions rely on healthy corneal epithelium, stroma and endothelium.
- Corneal transplantation (full thickness and/or lamellar) is currently the only treatment option for advanced corneal diseases.
- A global donor shortage entails a search of alternative treatments, which include corneal cell therapy.

Epithelial cell therapy

- The corneal epithelium regenerates from limbal stem cells. Their deficiency decreases visual acuity and causes blindness.
- Limbal autografting and cultivated limbal epithelial transplantation have been established to treat unilateral or partial limbal diseases with satisfactory outcomes.
- In cases of bilateral limbal diseases, other sources of epithelial cells (e.g., autologous conjunctiva, oral mucosa or allogenic limbus) have been used.
- Corneal epithelium generation from nonlimbal stem cell sources remains experimental, and mesenchymal stem cells have shown to be a promising cell source from *in vitro* and animal studies.

Stromal cell therapy

- Stromal scarring remains a leading cause of blindness worldwide.
- In recent years *in vitro* propagation protocols for the demanding corneal stromal keratocytes have been established.
- Different biological and synthetic substrates were successfully repopulated with keratocytes both *ex vivo* and *in vivo*.
- Corneal stromal stem cells have been identified and shown to differentiate to keratocytes.
- Different stem cell populations developed keratocyte characteristics *in vitro* and after intrastromal injection in animal models.

Endothelial cell therapy

- Corneal endothelial cells do not regenerate *in vivo*. Low endothelial cell counts are the main cause for corneal transplantation.
- Cultivated corneal endothelial cell injection is currently undergoing a clinical trial.
- Different biological and synthetic substrates are also being developed to facilitate cell transplantation.
- Nonocular stem cells have been induced to cells with corneal endothelial character but they have not shown any convincing results in animal studies.

Conclusion & future perspective

- The propagation and reimplantation of corneal cells is a promising approach to corneal cell therapy.
- Reliable and cost-efficient good manufacturing practice-compliant protocols, cell substrates and delivering techniques are the future challenges.

Conclusion

In recent years, there has been substantial progress in corneal cell cultivation and propagation. While CLET has been established to treat corneal epithelial defects, cultured corneal endothelial cells are undergoing the first clinical study. Cultivated CSKs could also have the clinical potential to treat corneal opacities. Other stem cell sources, however, lack reliable protocols to generate particular corneal cell types. The efficiency, stability, therapeutic outputs as well as ethical issues need to be clarified before further discussion to use in humans.

Future perspective

The human eye and in particular the cornea is an ideal organ for cell therapy, as it is easily accessible, avascular and immune-privileged. In addition transplanted cells are to some extent confined to the ocular tissue. The autologous transplantation of LSCs has been a great story of success. It can be anticipated that in the next 5–10 years clinical corneal cell therapy can be extended to CSKs and endothelial cells. To achieve this, reliable and cost-efficient GMP-compliant protocols, cell substrates and delivering techniques have to be established in cooperation with the regulatory authorities. Successful implementation of these primary cell therapies would also support the further integration of other stem cell sources, which to date still face technical and ethical issues.

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