

Eye Regeneration

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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), result-

ing 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

Matthias Fuest^{1,2},
 Gary Hin-Fai Yam^{1,3}, Gary
 Swee-Lim Peh^{1,3} & Jodhbir S
 Mehta^{*,1,3,4,5}

¹Tissue Engineering & Stem Cell Group,
 Singapore Eye Research Institute,
 Singapore

²Department of Ophthalmology, RWTH
 Aachen University, Aachen, Germany

³Eye-ACP, Duke-NUS Graduate Medical
 School, Singapore

⁴Singapore National Eye Centre,
 Singapore

⁵School of Materials Science &
 Engineering, Nanyang Technological
 University, Singapore

*Author for correspondence:
 jodmehta@gmail.com

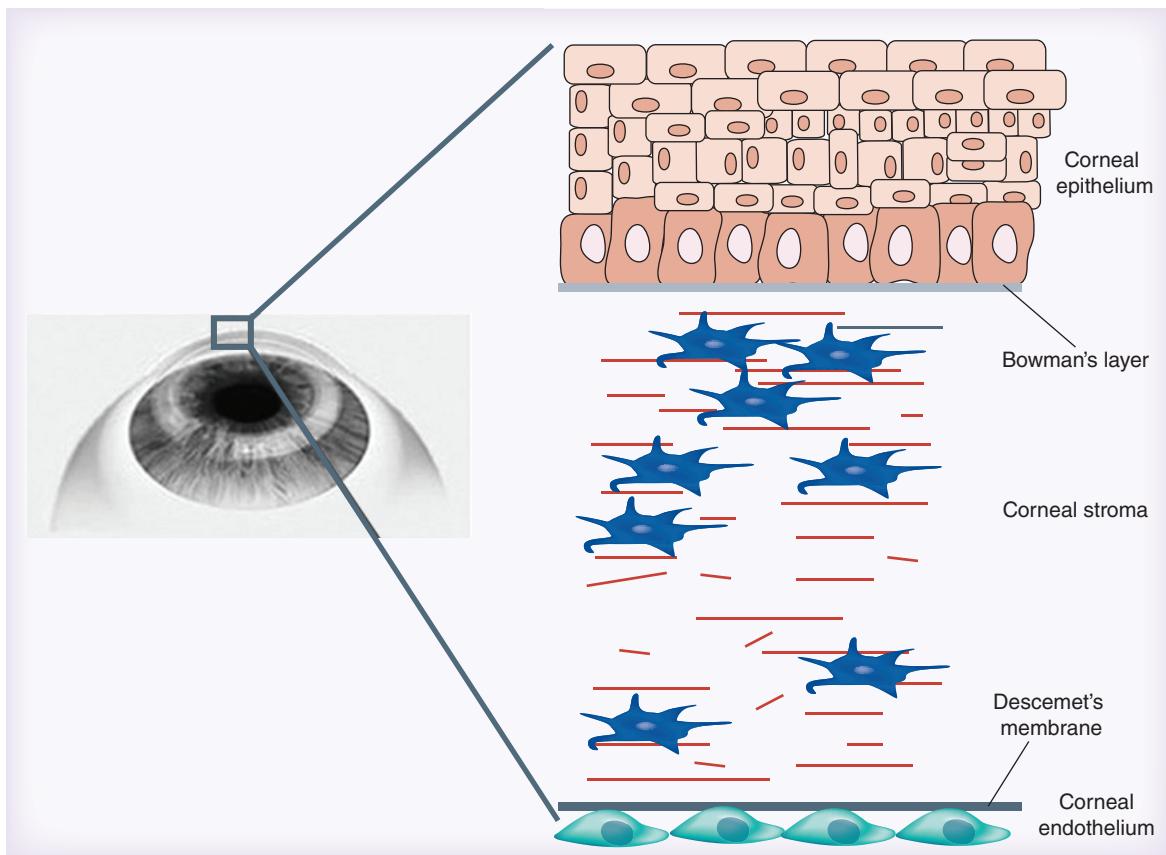


Figure 1. The human cornea consisting of five known layers, three cellular (epithelium, stroma and endothelium) and two interface lamellae (Bowman layer and Descemet membrane).

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 west-

ern 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

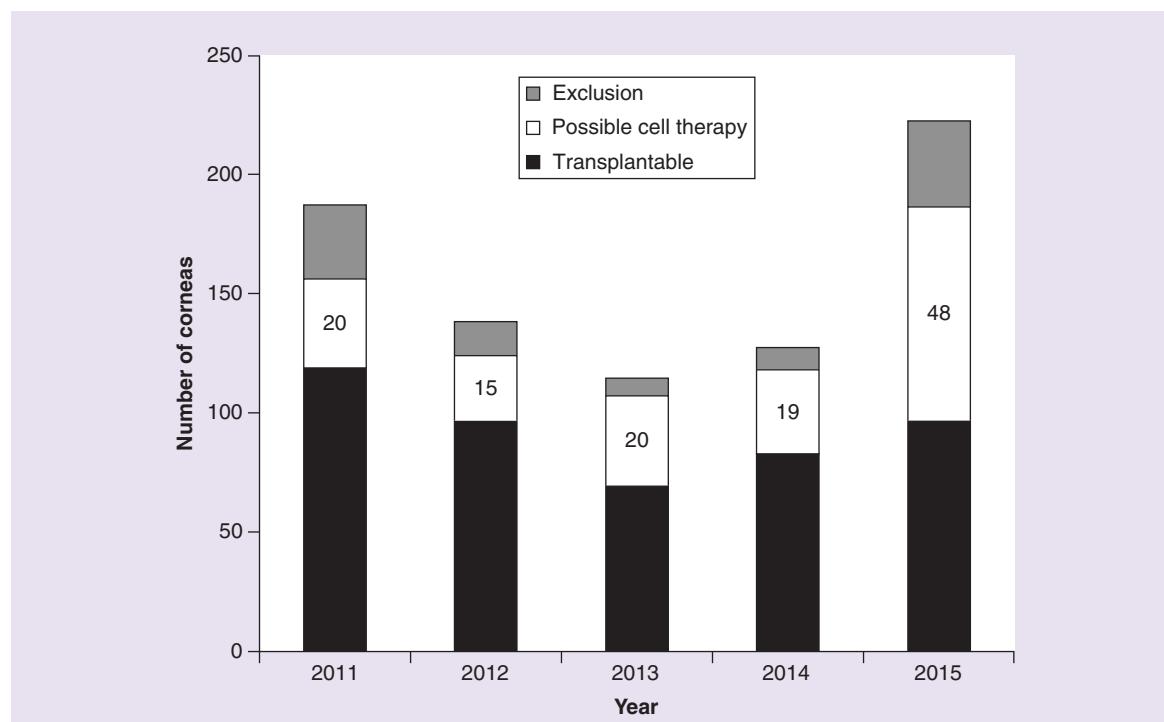


Figure 2. Corneas processed by the Singapore Eye Bank in years 2011–2015. Transplantable corneas had no limitations, excluded transplants (positive serology, contraindications, contamination, among others) had to be discarded, possible cell therapy corneas (percentage of all corneas displayed) had low endothelial cell counts, scarring, 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 LSCL, 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 LSCL 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 LSCL 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 Δ Np63 α was found to be important for

(A) Corneal epithelium

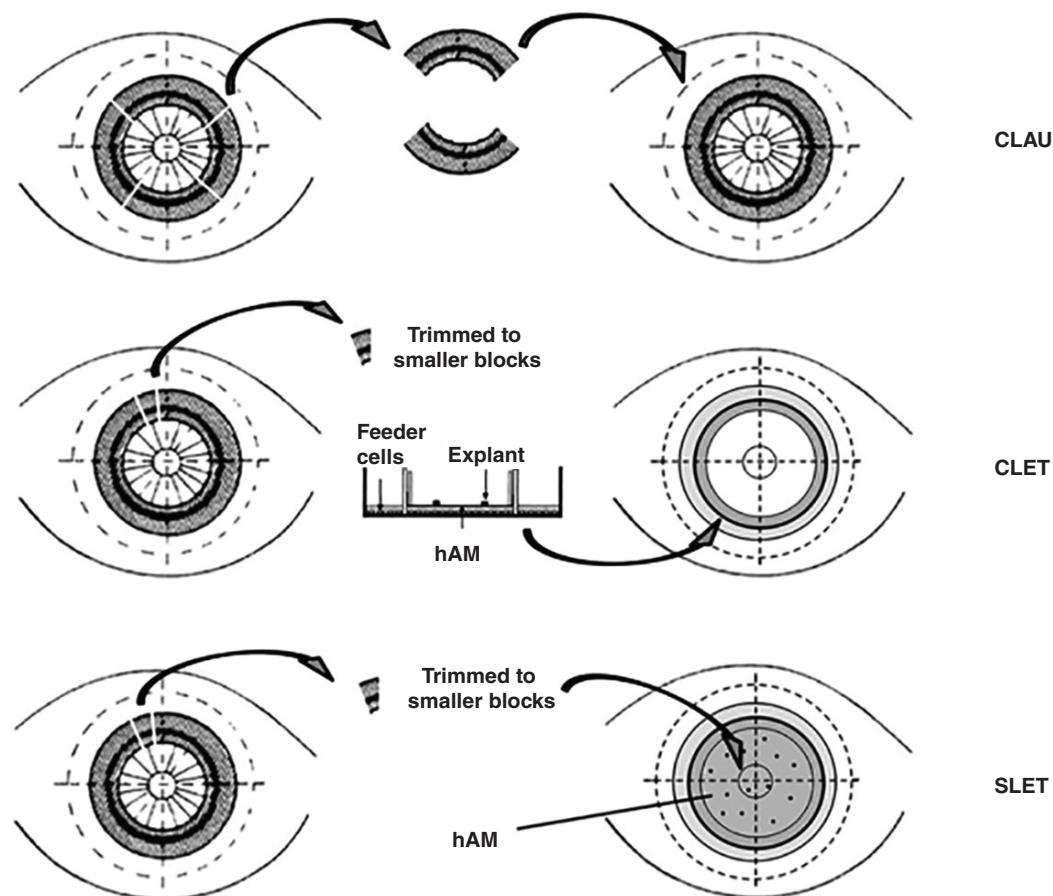


Figure 3. Corneal cell therapy. (A) Corneal epithelium can be replaced by the clinically established techniques of CLAU, CLET or SLET. (B) Corneal opacifications have experimentally been treated by intrastromal injections. In case of more advanced stromal damage, tissue can be replaced by stacking substrates 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 epithelial transplantation.

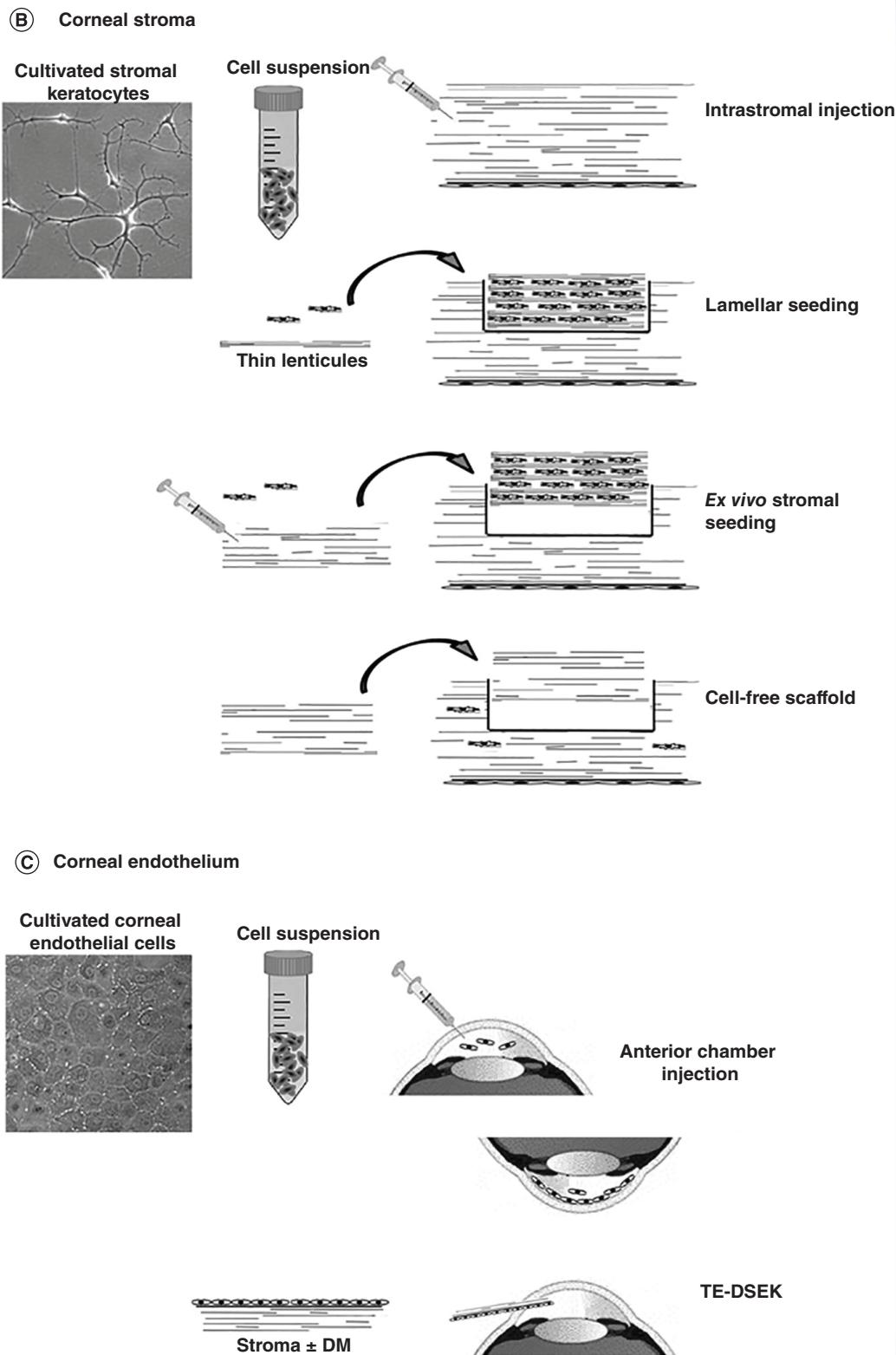


Figure 3. Corneal cell therapy (cont.).

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 (Δ Np63 α -positive) LSCs.

Bilateral LSCD

In cases of bilateral LSCD, where autologous LSC transplantation is impeded, other autologous non-ocular 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 β 1, vimentin, connexin43 and CK3/12 [38]. Transplan-

tation 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 Δ Np63 α 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 α 5-integrin and α SMA 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 tumorigenicity 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- β 3 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 lenticles, 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 lenticles 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 graft-

ing 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 $\text{Na}^{2+}/\text{K}^+$ 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/mm², 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- β 2 [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 PDLCs 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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Regulatory requirements in the good manufacturing practice production of an epithelial cell graft for ocular surface reconstruction

In the past decade, stem cell therapy has been increasingly employed for the treatment of various diseases. Subsequently, there has been a great interest in the manufacture of stem cells under good manufacturing practice, which is required by law for their use in humans. The cells for sight Stem Cell Therapy Research Unit, based at UCL Institute of Ophthalmology, delivers somatic cell-based and tissue-engineered therapies to patients suffering from blinding eye diseases at Moorfields Eye Hospital (London, UK). The following article is based on our experience in the conception, design, construction, validation and manufacturing within a good manufacturing practice manufacturing facility based in the UK. As such the regulations can be extrapolated to the 28 members stated within the EU. However, the principles may have a broad relevance outside the EU.

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An introduction to regulations

Regenerative medicine (RM) has been defined as the “process of replacing, engineering or regenerating human cells, tissues or organs to restore or establish normal function” [1]. RM promises to revolutionize patient care in the 21st century through an amelioration of cell and tissue replacement therapies. Stem cell research plays a dominant role in RM through translational research aimed at repair and regeneration of diseased or aging tissues and organs. A medicinal product, used for tissue regeneration within the EU, must comply to the European regulations on the production of human [2] and veterinary [3] medicines and pharmaceuticals. Within Europe, the manufacture of medicines is regulated by EMA, who decrees that each EU member state is required to elect a competent authority to oversee licensing of manufacturing facilities ratifying their compliance to good manufacturing practice (GMP). GMP is a quality

system for ensuring that medicinal products are manufactured consistently and to defined standards thus ensuring medicines are as safe as possible. GMP has historically been employed in the production of small molecules, proteins, vaccines and monoclonal antibodies [4]. However, in 2004, the European Commission ratified the Tissues and Cells Directive (EUTCD) [5] which reclassified gene therapy, somatic cell therapies (which includes stem cell therapy products) and tissue-engineered products as advanced therapy medicinal products (ATMPs) and imposed their manufacture (i.e., expansion or modification) to apply to the same GMP principals. Two directives lay down the principals and guidelines of GMP for medicinal products, Directive 2003/94/EC which is concerned with medicinal products of human use [6] and 91/412/EEC intended for veterinary use medicines [7].

One potential confusion within the field of cell therapy manufacturing is what regulations

Radhika Sheth-Shah^{*1,2},
Amanda J Vernon^{1,2}, Shankar Seetharaman³, Michael H Neale^{1,2} & Julie T Daniels^{1,2}

¹Cells for Sight, Transplantation & Research Programme, University College London Institute of Ophthalmology, London, UK

²Ocular Biology & Therapeutics Division, University College London Institute of Ophthalmology, London, UK

³Pharmanswers LTD, 13 Station Road, Fulbourn, Cambridge, CB21 5ER, UK

*Author for correspondence:
radhika.shah@ucl.ac.uk

applies to human tissue and/or cells isolated from tissue used for direct transplantation or as part of a medicinal product. As previously mentioned, the EUTCD was issued in 2004 and one of its aims was to clarify these definitions. Where whole tissues intended for human application, are donated from the living or deceased, the consent, procurement, processing, testing, storage and disposal of that whole tissue is governed by EUTCD with UK enforcement provided by the Human Tissue Authority (HTA). Where individual cells, isolated from whole nonembryonic human tissues, are expanded or modified to make a cell-based medicinal product then their manufacturing and quality control testing, would fall under the ATMP Directive 2009/120/EC [8] and the regulating authority would be the Medicines and Healthcare products Regulatory Agency (MHRA). The MHRA are the competent authority within the UK, whose remit covers the regulation of medicines, devices, blood and ATMPs for human application. In the UK, manufacture of veterinary products is regulated by the Veterinary Medicines Directive.

In addition to ensuring medicines are manufactured to GMP, the MHRA also issues authorizations allowing the distribution of safe and tested medicines on the market (termed as marketing authorization [MA]), or authorizes their use within a clinical trial, the medicine in this case is termed an investigational medicinal products (IMP). Facilities manufacturing medicines intended for release to the market must be granted a manufacturing license (MIA) by their competent authority, while facilities manufacturing IMPs must also obtain a separate MIA IMP license.

In the UK, in addition to the above-mentioned manufacturing licenses (MIA or MIA IMP), ATMPs may be manufactured under a UK 'specials' or a hospital exemption license for an unmet clinical need, that is, where no alternative licensed product is available. Cells for sight (CFS) have experience of manufacturing ATMP 'specials'. Here, a bona fide, unsolicited request from the patient's surgeon, in the form of a prescription, is required to commence the manufacturing process. The legal responsibility for use of the ATMP in this case rests with the surgeon. The main difference between the two types of license are that the holder of a 'specials' license can manufacture an ATMP for any EU member, whereas a hospital exemption holder may only manufacture ATMPs for their own hospital's patients. ATMPs manufactured as 'specials' must meet the expectation of 2003/94/EC [6] and Guidance Note 14 [9] and may be released by the quality control (QC) of the manufacturing facility as opposed to the qualified person (QP).

At CFS, we manufacture ATMP MIA IMPs generated from expended stem cells to produce novel cell-

based and tissue-engineered products. Medicinal products must be certified by a QP prior to release. The QP plays an integral role in medicine manufacture and must take final responsibility for the release of that product for human or animal use. The QP must ensure the product is safe by ratifying it has been manufactured to GMP and in accordance with the MA (for products released to the market), or in accordance with an IMP Dossier and product specification file (for IMPs).

Irrespective of the type of medicine being produced, and the type of license a manufacturing facility holds, the principles of GMP must always be applied in order to ensure medicines are consistently produced, of the highest quality, safe and effective for the end user. Under the EU regulations, ATMPs, which are classified as sterile medicinal products, must be manufactured within a cleanroom to ensure no contamination is transferred to the recipient. There are many requirements of a cleanroom in order for it to be 'fit for purpose' and the following section describes these in detail.

Cleanrooms

A cleanroom is a laboratory in which the level of particulate and microbial contamination is controlled.

In Europe, Eudralex Volume 4 Annex 1 recognizes four clean room grades (A–D) based on the maximum permitted number of particles per m³ and microbiological load limits [10].

Grade A is considered the critical zone for high-risk manufacturing that is, any open processing where the cells or media might be exposed to the environment or as a filling zone, stopper bowls, among others. Grade A environment is generally provided by a laminar air flow systems running with a homogenous air speed of 0.36–0.54 m/s. Particulate matter in a product is a concern with respect to patient safety and for this reason, the allowable particle level in the critical grade A zone is restricted to 3520 and 20 for 0.5 µm and 5 µm particles, both in-use and at-rest, respectively [11].

Grade B usually provides the background environment for the grade A area and is used for aseptic preparation. The maximum number of permitted particles in grade B is 3520 and 29 for 0.5 µm and 5 µm particles at-rest, respectively. In-use limits rise to 352,000 and 2900 for 0.5 µm and 5 µm particles, respectively [11].

Grades C and D are clean zones that can be used for performing the low-risk stages in the manufacture of therapies. As therapies are less likely to be exposed to the environment in grades C and D, the number of allowable particles is increased substantially and is 'not defined' while in-use, at the grade D stage [11].

The environmental conditions within the cleanroom are controlled by a sophisticated heating ventilation and air conditioning unit, which controls the

temperature, humidity, differential pressures between rooms and particulate content of the air. Clean air within the manufacturing facility is generated by a series of high efficiency particulate air (HEPA) filters that deliver pressurized air, via a dedicated air-handling unit, to each room. Air is filtered through a series of prefilters, panel filters and a terminal HEPA which is classified by its efficiency to remove its most penetrating particle size. At CFS the terminal HEPA is classified as H14 which means it removes 99.995% of particulates 0.1–0.3 µm [12]. A pressure differential of 10–15 Pa between successive rooms creates a clean air cascade, which ensures that the air grade of each room is maintained. This pressure differential also ensures that particles within each room are carried outward with the outward flow of air. Where microbial cross-contamination is not a concern, the air can be recirculated; however, due to pressure drops through natural seepage, supplementation by filtered fresh air supplied from an air handling unit (AHU) is required. The air exhausted from the cleanroom is returned to the AHU (or in the case of single pass systems vented to the outside). The exhaust may also be HEPA vented depending on the type of material (e.g., viruses or genetically modified organisms [GMOs]) being manufactured.

Generally, the airflow within the cleanroom is either turbulent or laminar. Laminar is unidirectional and involves a continual downward constant stream of air. Most cleanrooms are built with this design; however, some are turbulent flow which is not unidirectional and this method is used to move particles around the facility until it ultimately drives them to the floor and toward the extracts. Turbulent flow cleanrooms are allowed for cell therapies when manufacture is performed under a laminar flow cabinet or bench.

Where protection of the product is required, such as the cell therapy products manufactured at CFS there must be a positive pressure air gradient, whereby the air moves outward from the grade A/B laboratory in order to minimize environmental contamination of the product. For gene therapy products, or those containing high potency drugs, the primary processing grade A/B laboratory is maintained under negative pressure surrounded by positive pressure gradients for containment purposes. This is usually done through an air sink outside the processing room.

Construction of the GMP facility

From a regulatory point of view, premises must be designed, constructed, located and maintained to suit the operations to be carried out. Materials used to line the floor and walls should be impervious to liquids and not release particles. Tiles should be avoided as gaps between tiles allow space for contamination to settle

within and are also hard to clean. Internal surfaces should smooth and suitable for cleaning. The AHU should be situated outside the GMP facility so as to minimize the chance of contamination during its servicing and maintenance. Overall, the facility should be ergonomically designed to minimize the likelihood of operator error while allowing room for effective cleaning and maintenance so as to avoid build-up of particulates and cross-contamination that could adversely affect product quality.

Once the facility is structurally built, the internal environment must be established. For cell therapy facilities, HEPA filters are usually installed in the ceiling with an extract near the floor which ensures that the majority of the facility is always exposed to airflow. Prefilters are generally installed in order to reduce the particle load on the HEPA filters. Pre- and HEPA filters should be regularly serviced and checked to ensure that no particulate matter makes its way to the cleanroom facility. For optimum performance, pre- and HEPA filters should be changed as per the manufacturer's instructions. Lifespan of these filters predominantly depends on the surrounding environment for example filters in an urban area might get dirtier compared with filters installed in a facility based in a rural landscape.

Typically, the preferred method for entry/exit within the cleanroom is the one-way flow of personnel, consumables and waste as it prevents room re-entry, therefore, minimizing the risk of cross-contamination. Exit occurs through a separate degowning room. However, this setup is not always possible; therefore, backtracking through the previous rooms is allowed, albeit with extra precaution. Pass through chambers are generally built within the walls to allow for transfer of consumables, reagents and waste through the facility. Both the product and the waste can be transferred through the pass through chambers, however, they should be physically segregated.

Qualification processes in the validation of a cleanroom

Cleanrooms have to be validated and undergo a process of qualifications (Figure 1). Only once it has passed all these requirements, can it be licensed for use in the manufacture of ATMPs destined for human application.

Equipment

Only equipment necessary to manufacture the medicinal product should be installed within the facility. At the outset, equipment should be validated, either by the supplier or the manufacturing team, to ensure it is fit for purpose and does not compromise product quality. The equipment should be placed in such a way that it does not obstruct the airflow within the facil-

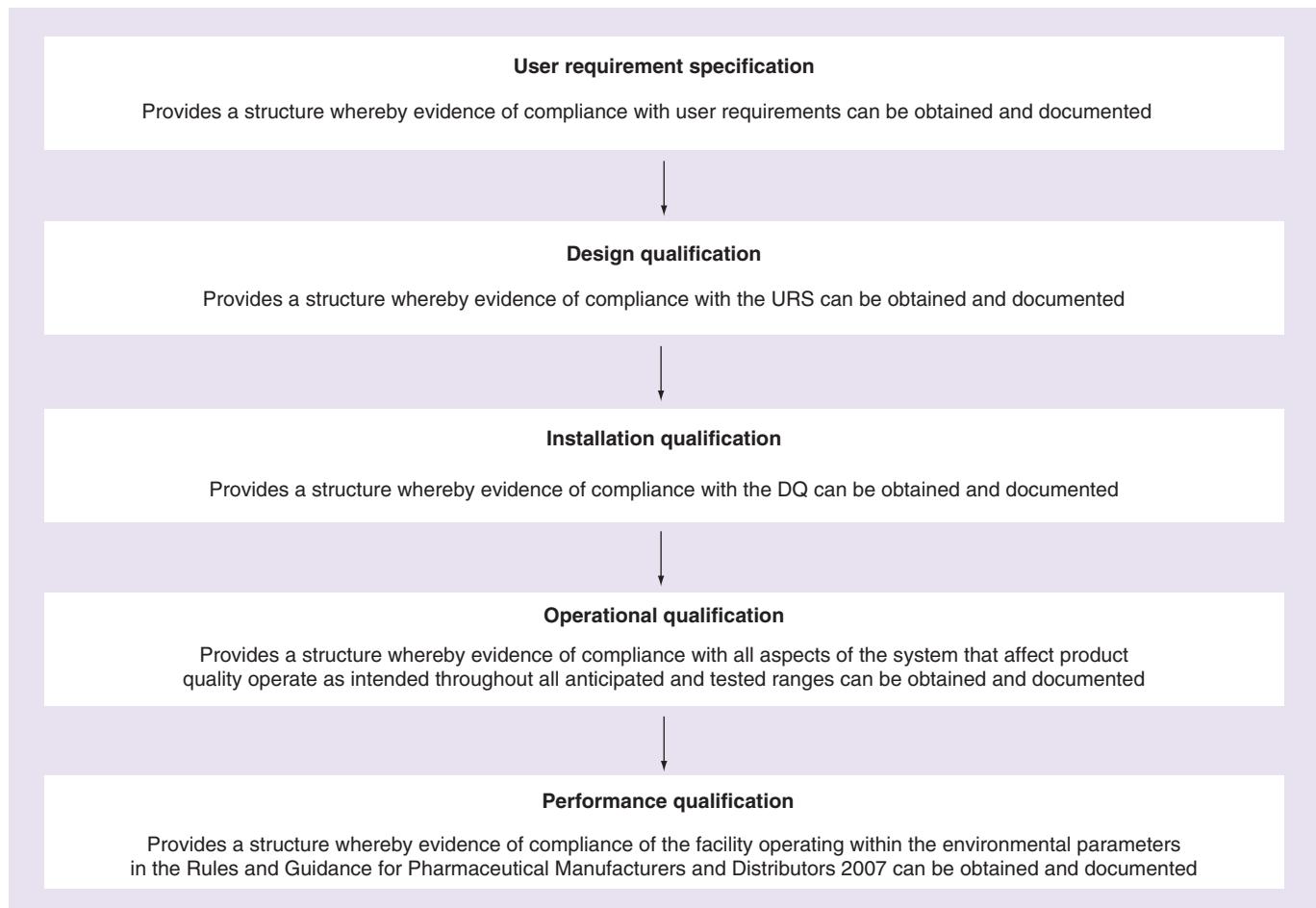


Figure 1. Validation of the cleanroom. Validation of the cleanroom will include a URS which is a high level requirement and expectations for systems to be used as the basis of testing during the performance and/or operational qualification phases. The URS leads to a DQ which is a document that defines all hardware and software components to be installed and the configuration management plan needed to meet the URS and/or owned project requirements. This in turn would lead to an installation qualification which is the documented verification that all aspects of the system that can affect product quality adhere to approved specifications and are correctly installed. The cleanroom can be commissioned following the installation qualification. This involves a well-planned and systematic verification of the cleanroom using inspection, testing and documentation to ensure that the facility, system and/or equipment is constructed, installed and started and functions appropriately, as per the design and installation requirements and acceptance criteria. After commissioning, the operating qualification follows which is the documented verification that all aspects of the system that can affect product quality operate as intended throughout all anticipated and tested ranges. Finally the performance qualification is performed which is the documented verification that all aspects of the system that can affect product quality repeatedly and consistently perform as intended, meeting predetermined acceptance criteria.

DQ: Design qualification; URS: User requirement specification.

ity. Postinstallation, all equipment must be serviced and calibrated as necessary to ensure that the quality of products is not compromised. Details of equipment specification, performance criteria, servicing and calibration must be retained for future reference.

Personnel

Of importance to every GMP facility are the personnel who physically manufacture the product as per written procedures. Therefore, it is essential that there are sufficient personnel available to carry out all the tasks required for manufacture of the product. All personnel should be aware of and trained to their specific tasks.

Training records for each individual should be generated and filed. Competence for all GMP-related tasks including cleaning, gowning, aseptic techniques, among others, should be documented on the training record.

Each facility should have an organizational chart in which the responsibilities and the relationships between each individual are explicitly defined. Most GMP facilities are divided into two departments: production and quality control/assurance (QC/QA); each as independent from the other as possible [13]. The production team is trained in and is responsible for all aspects of manufacturing the product. The QC department does not get involved in processing

operations but does monitor and review the quality management system (QMS), approve starting materials, ensure testing and validations are carried out and reviews product-related documentation/investigations. The QC department may have a QA unit which may perform all, or specific, aspects of the above dependent upon the companies' organization. In smaller facilities, both the QC and QA responsibilities maybe managed by the same individual.

Facility monitoring

The facility and equipment should be regularly monitored to ensure that there is minimal risk to the product quality. Ideally, an automated system should be installed to ensure continuous monitoring of all parameters. This should be supplemented by manual monitoring to ensure that any fault within the automated system is promptly detected without any effect on the product quality. Various parameters need to be monitored to ensure that the facility and the equipment housed within are functioning within their set specifications, these include but are not limited to: room temperatures and humidity, room pressures, pressure differentials, particle levels within the facility and safety cabinets, temperature, CO₂ and humidity of the incubators, temperature of the fridge/freezers, among others. Each parameter should be continuously monitored and should additionally be manually checked during working hours.

Monitoring particle counts is extremely important as particles may serve as a vector for attachment of various microorganisms which pose a risk of contamination to the product. Particles, by themselves, can also contaminate the product. Related to the particle counts are the pressure differentials as a positive pressure cascade ensures that particles are removed from the GMP facility in an outward fashion. Equipment parameters are important as they maintain the integrity of the product and/or reagents.

Maintenance of the cleanroom

Following the qualification process, cleanrooms must be maintained to ensure they are 'fit for purpose'. This includes ensuring personnel are trained to cleanroom procedures and ensuring environmental monitoring and cleaning is routinely performed.

Cleaning

A documented cleaning routine is used to minimize the risk of particulate or microbiological contamination of products resulting from airborne and surface contaminants. The effectiveness of the cleaning routine is determined by regular environmental monitoring according to a documented series of validated proce-

dures. Cleaning includes all surfaces, equipment, consumables, doors, walls and floor. It should be performed by trained personnel and be thoroughly documented.

At CFS, cleaning of the manufacturing facility is performed on an in-use basis, a weekly basis, a monthly basis and a quarterly basis. 'Critical areas', that is, those directly involved in product processing are cleaned on an in-use basis. 'General areas', that is, those not directly involved in production but which present a low risk of contamination to critical areas via transfer, are cleaned with sterile 70% isopropyl alcohol (IPA) according to a weekly, monthly and quarterly schedule, with alternating biocide A (bactericidal and fungicidal), B or C (bactericidal, fungicidal and sporicidal virucidal) cleans on a monthly basis. If environmental monitoring results indicate that additional measures are required then extra *ad hoc* cleaning with biocide A, B or C is performed. Biocides must be alternated to avoid development of resistance within organisms. Deep cleaning for example on an annual basis or following a persistent contamination resistant to routine cleaning may be performed with vapor hydrogen peroxide. To minimize particles within the facility, cleaning reagents are sprayed onto sterile low particle shedding wipes and then the reagent is wiped onto the surface being cleaned using unidirectional overlapping strokes to ensure that the whole area is cleaned. Ceiling, walls and floors are cleaned with a mop head attached to a mop. Other facilities may use preimpregnated wipes for surfaces and equipment or other validated cleaning systems. IPA is allowed to evaporate over time while biocides are left in contact with surfaces for 5 min. Some biocides leave residues, and for these a second clean with IPA is performed. Biocides may be corrosive by nature, therefore, care should be taken to ensure that the biocide does not dry out and leave behind any residue.

Large facilities tend to use preimpregnated wipes for surfaces and equipment. This is advisable for large facilities as it is more cost effective whereas in smaller facilities, the impregnated wipes may dry out if left opened for days, leading to waste of reagents and money.

Environmental monitoring

To establish whether the cleaning protocols meet the required standards with respect to control of particulate and microbiological contamination within the production areas, routine environmental monitoring is performed according to documented procedures. This is performed both 'in-use' to determine the level of contamination present during production, and 'at-rest' on a weekly basis to determine the effectiveness of cleaning routines. Environmental monitoring (EM)

utilizes a number of agar plates to monitor bacterial and fungal contamination, at CFS, tryptone soya agar and Sabouraud dextrose agar plates are utilized respectively for this purpose. The plates themselves are of two different types: settle and contact (Figure 2). Contact plates themselves are used either to perform contact monitoring or active air sampling.

Settle plate monitoring is a direct method for evaluating the approximate number of microorganisms depositing on the agar surface in a set amount of time from air within the cleanroom.

Contact plate monitoring is a method of surface sampling performed in order to determine the number of microorganisms/contamination on any surface within the cleanroom or upon the operator's apparel. Contact plates normally have a raised media surface to allow for direct application to the test surface.

The air circulating within the cleanroom is also monitored by an active air sampler which captures air through a high pressure inlet. The captured particles collide against the agar surface and settle on it.

At CFS, EM plates are incubated at specified temperatures (35°C for bacteria and 22°C for fungi) for 3–5 days and checked for colony forming units to determine the number of microbe bearing particles deposited onto the agar. Positive and negative controls are also prepared to ensure the accuracy of results. Results from 'at rest' and 'in-use' environmental monitoring are recorded in a spreadsheet in order to determine any contamination trends. This information is used to determine if there is a need for corrective and preventive action, according to whether action levels or alert levels have been reached. Action levels are indicative of unacceptable levels of microbiological contamination and require corrective and preventive action to be taken in accordance with written procedures. Action levels are those defined in 'Rules and Guidance

for Pharmaceutical Manufacturers and Distributors 2007' [11]. Alert levels are set at 50% less than action levels. Upon exceeding an alert level, appropriate corrective and preventative actions are performed.

Training

Personnel

Personnel are also a significant source of contamination. Therefore, traffic of personnel, visitors and engineers is strictly controlled to minimize the risk of particulate and microbiological contaminants entering the manufacturing facility. Cleanroom entry, gowning and hygiene routines are implemented according to validated procedures, in which all users of the facility are trained. Visitors and engineers are given training appropriate to their task prior to entry within the cleanroom.

All users of the facility are also required to adhere to some basic behavioral and personal disciplines in order to minimize particle shedding and potential contamination, these include:

- Maintenance of a good level of personal hygiene, including showering regularly, keeping dandruff at bay and moisturizing dry skin to reduce skin shedding;
- No cosmetics, talcum powders, hair sprays, nail polish or similar materials;
- No jewelry or watches within the cleanroom;
- Controlled movements within the cleanroom, with no rushing and to refrain from any sudden/jerky movements.

In terms of particle shedding, movement is perhaps the greatest factor as the number of particles shed is proportional to the level of activity performed, with

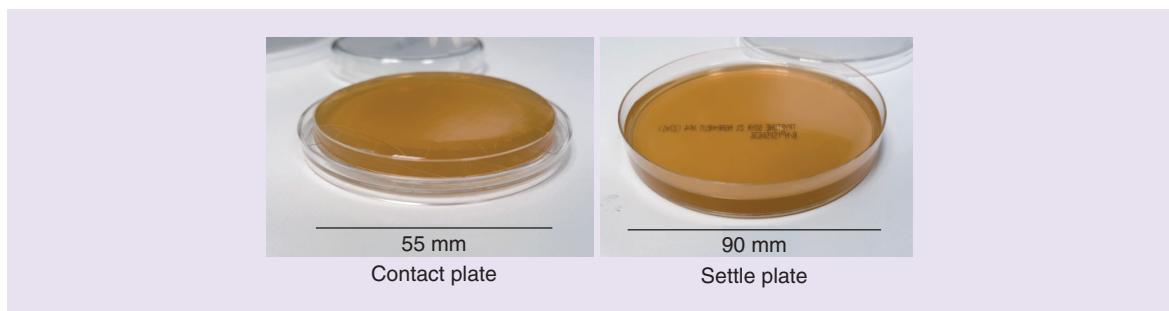


Figure 2 Examples of a contact and settle plate for environmental monitoring. At cells for sight, both plates are filled with either tryptone soya agar, or Sabouraud agar depending on the requirements of the monitoring. The agar also contacts various chemical neutralizers to inhibit the action of biocides to prevent false negatives. The contact plates have a raised agar surface to allow monitoring of surfaces without the side of the dish preventing contact being made (such as cabinet surfaces or glove prints form operators) whereas the settle plates are used for passive monitoring of the air within the cleanroom. Additionally, the contact plates are used in conjunction with an active air sampler to actively monitor air contamination when required.

100,000 particles $\geq 0.5\text{ }\mu\text{m}/\text{min}$ shed while sitting, rising to 1 million when moving and 5 million when walking [14]. Therefore, slow, controlled movements within the cleanroom facility are essential to minimize aberrant particles.

Gowning

Outdoor clothing should not be brought into the facility beyond the grade C/D changing room. For wearing within the grade A/B areas, sterilized protective garments should be provided (Figure 3). Personnel must be trained to change clothing in accordance with a gowning procedure in order to minimize particle shedding and potential contamination within the facility.

At CFS, all garments are sterilized by gamma irradiation by a specialist laundering company and are delivered triple wrapped. Each garment is single use and is sent back to the laundering company to be cleaned and resterilized a finite number of times after use.

In order for an operator to perform any processing in the facility they need to have passed a gowning training test, this involves gowning in according to cleanroom techniques to enter the grade B laboratories. Gowning is followed by a series of microbial monitoring contact plates to assess levels of contamination on areas of gowning which may come into contact during the gowning process – gloves, forearm, forehead, chest and stomach. If the plates have passed that is, no colony forming units on the contact plates, the operator is allowed to process in the facility.

Aseptic training

All staff members who work in the manufacturing facility, including those involved in product manufacture are trained in aseptic technique on recruitment and at regular intervals thereafter.

At CFS, all operators are required to pass a training module to demonstrate aseptic technique within the biological safety cabinets (grade A environment). This involves a 3-day process of manipulating cell culture plates or flasks and liquid handling. This is performed by transferring sterile tryptone soya and Sabouraud dextrose broths between wells on three separate days. A sample from these broths is further inoculated in independent tryptone soya and Sabouraud dextrose broths and incubated for 3–5 days. Post incubation, the broths are analyzed for turbidity, with turbid broths indicating a contamination, for example, failure to perform the training aseptically. Additionally in-use monitoring is also performed assessing the working environment and the operators' gloves for contamination. In order to pass this training module, all broths should be free from turbidity and monitoring plates should be below allowable limits [11]. This training module is

repeated every 6 months and results recorded in the operators training record. This procedure is specific to CFS, other facilities will need to simulate training appropriate to the processes to be performed within their respective facilities.

Quality management systems

A quality management system (QMS) has the purpose of assuring product specification and quality through the control of interconnected elements and to mitigate potential risks to the product.

QMS is based upon ISO 9001:2008, a set of standards originally developed in 1987 to maintain QA in product manufacturing and service industries. The pharmaceutical industry has further developed these standards through the ICH, to produce a harmonized comprehensive model for an effective pharmaceutical quality system designed for the entire product lifecycle.

Within the pharmaceutical industry, a QMS is the requirement of Directive 2003/94/EC (manufacture of medicinal products for human use); [6] and Directive 91/412/EEC (manufacture of medicinal products for veterinary use); [7] and ensures the principals and guidelines of these directives are implemented, including compliance to GMP.

All further reference to the QMS within this publication will be based on the CFS QMS, unless otherwise specified. The QMS defines how the activities performed during ATMP manufacture ensure compliance to local regulatory requirements of the MHRA and HTA. The CFS QMS is organized into the following categories: validations; donor recruitment and patient treatment; production and QC; QA, where each category comprises of a set of standard operating procedures (SOPs) that provide a top-level description of how the QMS is implemented (Figure 4).

Additional to the SOPs, various documented procedures (including QA forms, training forms, corrective and preventive action procedures and production protocols) are used to execute the QMS for the manufacture of ATMPs.

The following describes the requirements and the implementation of the various sections of the QMS and how it interacts with GMP compliance and human tissue regulations.

Validations are required to be performed for a process, reagent, a piece of equipment or for the manufacturing facility itself in order to ensure the parameter in question is 'fit for purpose' and will not affect product quality once initiated or implemented. Following quality approval, successful validations allow the parameter in question to then be built into the QMS through updates to existing GMP documentation or implementation of new GMP procedures.



Figure 3. Garments required to operate/process at or above grade B. Operators are required to remove outdoor shoes and clothes and change into cleanroom tunics, trousers and clogs, and the donning of gloves and a mobcap (not shown above) prior to entering the facility. A second level of gowning is then performed prior to the operator moving to the grade B laboratories, this involves putting on fresh gloves, a hood, safety glasses, a face mask, coverall and overboots, all of these items will have been sterilized by irradiation.

A section of the QMS is dedicated to Donor Recruitment and Patient Treatment to ensure the CFS facility, when manufacturing ATMP's using autologous or allogeneic donor tissue and cells, complies to the legal framework (Human Tissue Act 2004 [15], the Human Tissue Regulations 2007 [16] and EU Tissues and Cells Directives [5]) that dictates procedures surrounding consent for tissue donation, procurement, testing, storage, disposal and use of body parts, organs and tissue.

The QMS also details policies around production and quality control activities. The production SOP documents the required production procedures to be per-

formed to maintain GMP standards. Such procedures include: facility clean, facility and environmental monitoring, equipment planned preventative maintenance and QC sterility assessments for aliquoted reagents.

QA is directly concerned with assuring the quality of a product through controls that are described in a set of SOPs. Quality Assurance is also concerned with monitoring procedures and processes, providing a feedback loop to aid improvements in order to prevent error in the future. Although each of the aspects detailed in Figure 3 is essential to the smooth running of a cleanroom, only the critical sections will be discussed here.

The investigation of deviations forms a major part of any organization's strategy for risk management and quality improvement. Where a deviation or nonconformance occurs within a prescribed process, the incident needs to be recorded and corrected. The term 'deviation' is used to include process deviations, planned changes, out of specification events and any other noncompliance with GMP. This overall system includes recording of the deviation, assessment of the deviation in terms of risk to the facility, product or patient, root-cause analysis, planning and implementation of corrective and preventative action and routine trending of deviations so that higher levels of preventative action can be taken.

A complaint is defined as any defect in a product or service reported by either an external or an internal customer. The complaints and recalls SOP defines the process employed which includes risk and impact assessment, root cause analysis and implementation of corrective and preventative action to prevent a similar issue occurring in the future.

Change control is the process that defines the way in which changes to all aspects of the QMS are managed. In practice the following require change control: starting materials, documents, equipment, processes, any change that has a regulatory impact for example, key staff changes, new product types, facility changes and any other change that may affect the quality of the product. Changes can occur in response to many parameters for example, deviation, complaint, audit, introduction of a new project or contract change. Change requests are trended and reviewed periodically to help identify potential improvements.

A service level agreement must be in place when an organization provides a service to, or is provided with a service from, a third party. The service level agreement will establish the expectations and requirements that should be met by both parties. Should we define the different one?

Before a medicinal product can be used to treat a patient, it must be formally released for use by CFS

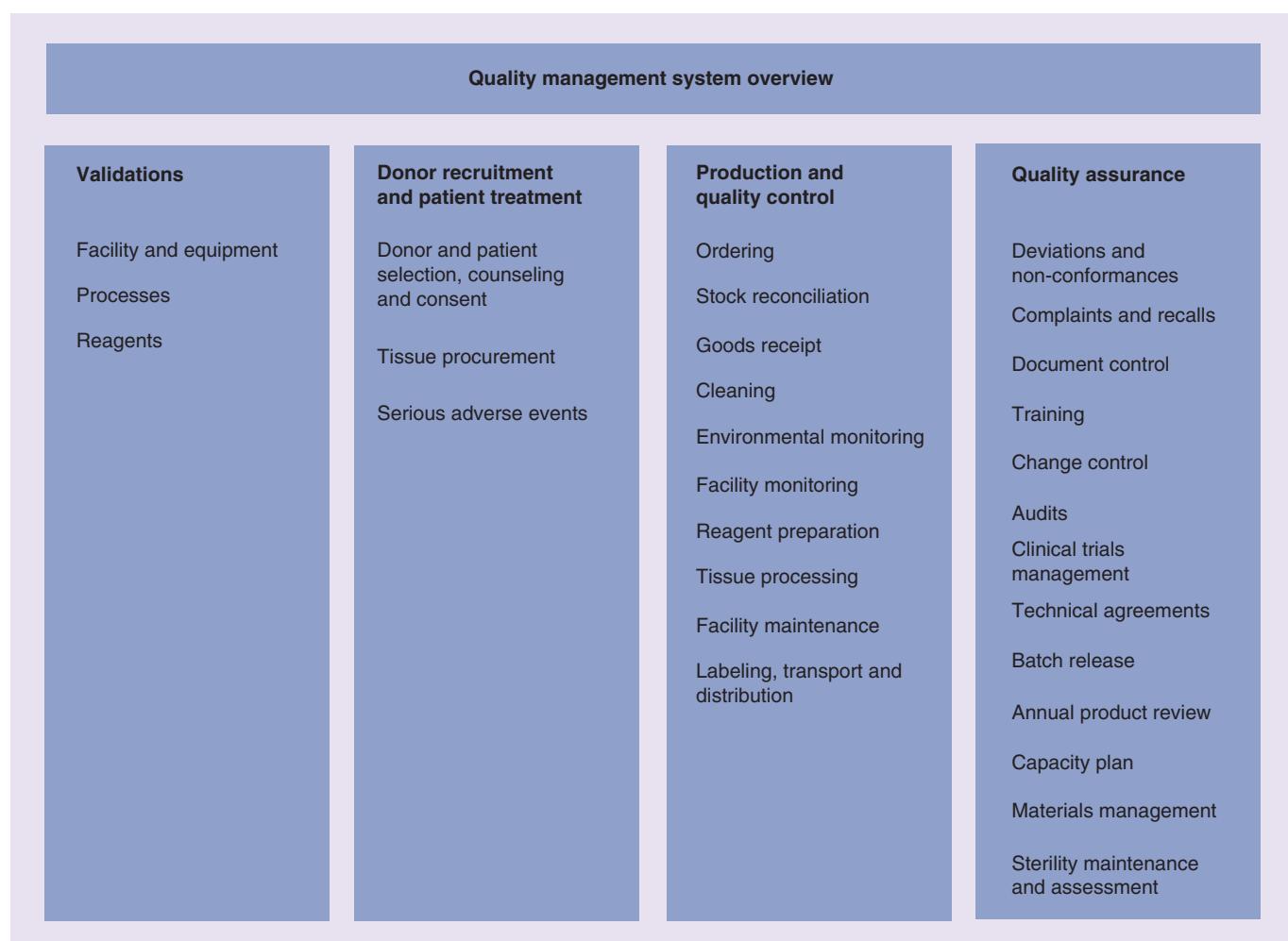


Figure 4. The cells of sight quality management system. The cells of sight quality management system is divided into validations, donor recruitment and patient treatments, production and quality control and quality assurance.

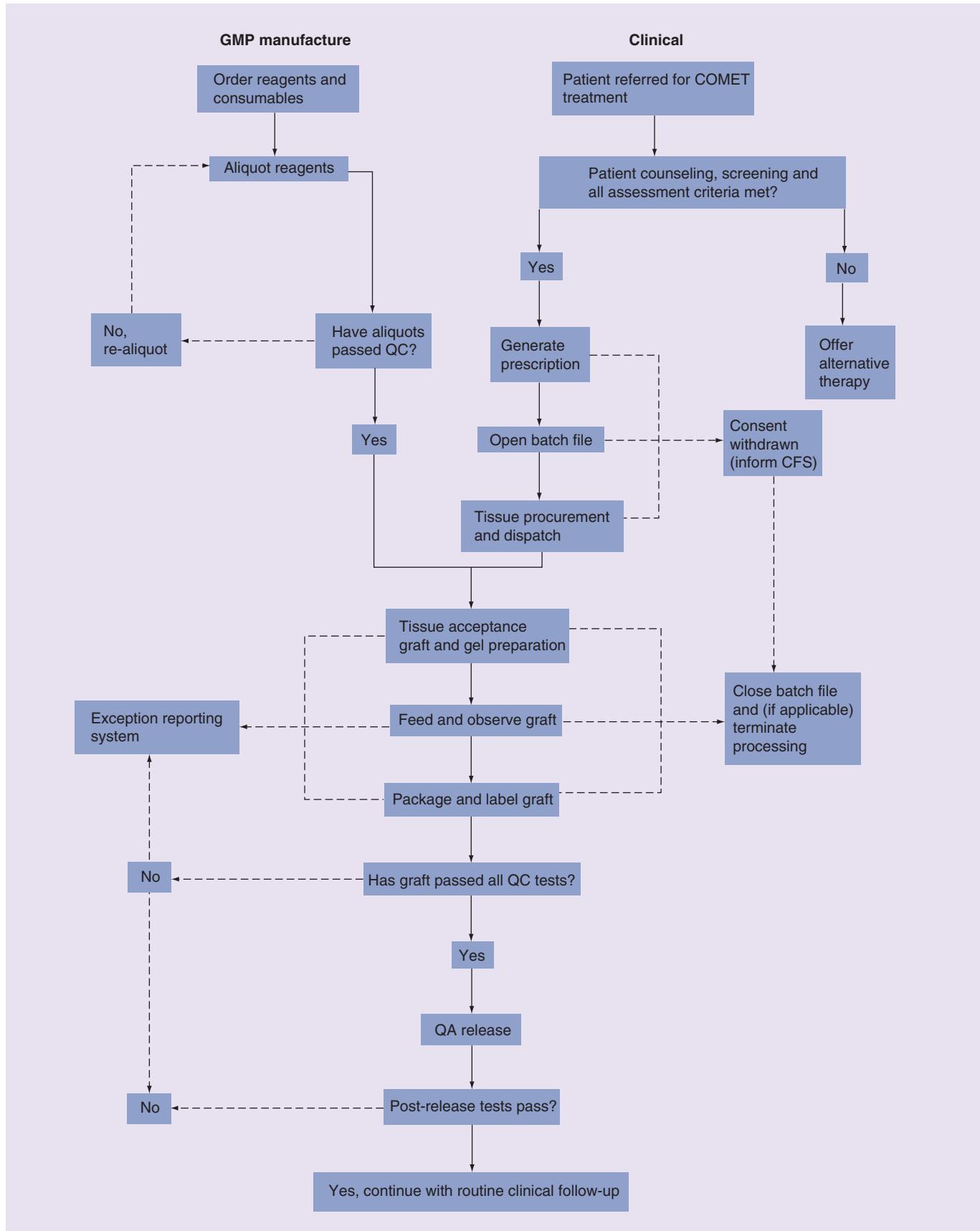


Figure 5. Process flow for the production of cultured epithelial cell grafts at cells of sight (see facing page). The process flow diagram illustrates the steps involved in the production of cultured epithelial cell grafts at CFS. It encompasses all the quality, production and clinical requirements essential in the production of a GMP compliant cell therapy graft.

CFS: Cells of sight; COMET: Cultivated oral mucosal epithelial cell transplantation; GMP: Good manufacturing practice; QA: Quality assurance; QC: Quality control.

and therefore only officially released products can be used to treat a patient. The Batch Release SOP documents the procedures by which a product is released for patient use including; review of batch records, test results, deviation reports and monitoring results culminating in a formal sign-off by the QP for IMP products or the QA manager for 'specials' products.

The materials management SOP documents the controls in place to mitigate potential risks to the product due to materials used within GMP manufacture. The controls described within this SOP include the following; goods/supplier selection and risk assessment, transmissible spongiform encephalopathy (TSE) compliance assessment, goods ordering and receipt, traceability and stock rotation/storage/reconciliation.

As previously described, all of the above factors have to be stringently controlled in order to ensure medicines (including cell therapy products) are manufactured safe, effective and of the highest quality. Below is an example of how a CFS product is manufactured in adherence with EU regulations and the CFS procedures described above.

Limbal epithelial stem cells & limbal stem cell deficiency

CFS have worked with academic, industry and National Health Service partners in the manufacture of cell therapy products. One particular cell-based therapy manufactured by CFS is used to treat patients' suffering from blinding eye diseases such as limbal stem cell deficiency (LSCD) which results in corneal degeneration.

The cornea is the clear, front surface of the eye that transmits light to the retina to enable vision. Corneal epithelium is separated from the conjunctival epithelium by a narrow band of tissue known as the limbal epithelium. Limbal epithelial stem cells reside within the limbal epithelium and play a role in the maintenance and renewal of the corneal epithelium [17]. Dysfunction or damage of the limbal epithelium can lead to either unilateral or bilateral LSCD, which is characterized by vascularization, conjunctival ingrowth and inflammation resulting in visual impairment [18]. LSCD can be treated by replacement of the lost or damaged limbal epithelial stem cells by autologous or allogeneic transplantation of limbal tissue. CFS devised a technique utilizing a fully reproducible fibrin gel substrate for the culture of autolo-

gous or allogeneic limbal and autologous oral mucosal epithelial cells [19].

Described below is a brief explanation of the production of a GMP compliant cell therapy graft within CFS.

Graft preparation

As previously described, the implementation of any process within the cleanroom requires validation. At CFS, the graft production process was conceptualized and optimized in the research laboratory before finally, performing a process validation within the cleanroom facility. The process of graft production within the cleanroom is described below. The flow of the production process can be observed in Figure 5.

Prescription & patient selection criteria

The process is initiated when the CFS team receives a prescription from the clinician requesting a specific 'specials' product. The clinician obtains patient consent, and with them completes donor selection and testing forms which include lifestyle and medical questionnaires and also includes testing at minimum for HIV-1 and -2, hepatitis B and C and syphilis [20]. There is generally no difference in virology testing between autologous or allogeneic donors so as to ensure that tissue coming into the cleanroom facility is identically screened. An exception would occur where patients have traveled to 'at-risk' countries or where the behavior and lifestyle questionnaire indicates further testing maybe required as per the guidelines. The results of a blood sample for serological screening may be obtained in sufficient time, prior to tissue donation, to allow assessment of infection risk to the GMP facility. In the UK, a second blood sample for serological screening should be obtained on the day of tissue donation (or within 7 days postdonation if the former is not possible) in accordance with HTA Direction 003/2010 [21].

Cell culture media

As there is no universally accepted list of suitable materials and reagents for the manufacture of ATMPs, each facility must perform a risk assessment (RA) to ensure that the components used pose the least risk to the patient. RA addresses various criteria including tests for viruses, bacteria, fungi, mycoplasma and endotoxins to justify the use of the product. Currently available cell culture reagents are mostly approved for research

use only. Therefore, the RA must demonstrate why the reagents in cell culture media are justified for use, as per the regulations. Manufacturers should provide all quality certificate available (i.e., Certificates of Analysis, origin and TSE/bovine spongiform encephalopathy compliance) upon which, these assessments will be made.

Cell culture process

Cell culture media for clinical grafts were prepared on a weekly basis to avoid any contamination during the cell culture process. Therefore, all the media components need to be aliquoted and pass QC prior to the start of cell culture. Once all the media components have passed their sterility testing, a limbal biopsy (autologous or allogeneic) or an autologous oral mucosal biopsy, as determined by the consulting clinician, may be accepted to commence cell therapy manufacture.

In the past, CFS manufactured epithelial cell grafts for patients using human amniotic membrane as described in [22]. However, due to batch-to-batch variability in amnion [23], CFS developed an alternative technique utilizing a CE-marked fibrin substrate for culture of epithelial cells [19]. This technique is currently being used by CFS for manufacture of epithelial cell grafts for ocular surface reconstruction.

The tissue culture process is set up as previously described in. The cells are fed with freshly prepared media every 2 days. Cell morphology, percentage of confluence and media turbidity are recorded at every feeding session.

Critical quality parameters

To release the epithelial cell graft, critical quality parameters must be met. These include but are not limited to adherent and healthy epithelial cells with cobblestone morphology, integrity of the fibrin gel, no contamination.

The graft is released for transplantation only if all of the above criteria are met. The release is authorized by the QA manager who reviews all of the batch records and associated testing results.

Packaging the graft

The cell culture media on the graft is replaced by transport media, in our case L-15, which supports the cells in environments without CO₂ supplementation, the culture plate has an adhesive primary label attached to it and the graft is packaged in a clinical pouch sealed with GMP compliant tape. The plate is then placed into a labeled transport box. The labels and the pouch are marked with details of the patient, the cell therapy product type, expiry date and expiry

time of the graft and facility contact details. Upon completion of the batch release process, the graft is delivered to the hospital for transplantation onto the patient's eye. Upon exchange of the graft to hospital personnel a 'chain of custody' form is completed to document the final stage in the process where the graft becomes the clinical teams' responsibility from this point.

Post release sterility data

The final media sterility data are only available post-transplantation. In the event of a positive result, the hospital and the MHRA should be alerted, as per their guidelines. The microorganism would be identified and the species communicated to the clinical team who would treat the patient as per the clinicians' directive.

Patient-related data storage

All documentation should be securely stored for the period of time defined by the directives under which the work is being performed.

Future perspective

Stem cell research has the potential to deliver a host of new cell therapies. The EMA has recently granted MA to the first ever commercial stem cell therapy to treat LSCL. With increasing research progressing toward clinical trials, there is an ever increasing need for an understanding of the regulations in the production of cell therapies. Due to the vast range of possible cell therapies, it is difficult for regulators to provide guidance on specific developments within the field. Reviews like this are likely to be beneficial for future manufacturing facilities and users as they would bridge the gap between understanding and implementing the various regulations and guidelines involved in the manufacture of cell therapies. This review is also beneficial for researchers planning ATMP manufacture or clinical trials to understand the additional requirements in transferring the cell culture process from the research laboratory to the GMP facility.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Executive summary

Regulatory requirements within the UK

- For manufacturing cell therapies, compliance to various legislations announced by the EMA is required. The most important directives describing the manufacture of cell-based medicinal products for human use are Directive 2001/83/EC, Directive 2004/23/EC and 2003/94/EC.
- Where whole tissues intended for human application are donated from the living or deceased, the consent, procurement, processing, testing, storage and disposal of that whole tissue is governed by European Commission ratified the Tissues and Cells Directive with UK enforcement provided by the Human Tissue Authority.
- Where individual cells, isolated from whole nonembryonic human tissues, are expanded or modified to make a cell-based medicinal product then their manufacturing and quality control testing, would fall under the Advanced Therapy Medicinal Products Directive 2009/120/EC and the regulating authority would be the Medicines and Healthcare products Regulatory Agency.

Good manufacturing practice facility construction & maintenance

- The good manufacturing practice facility should be constructed in a way that it is easy to maintain. The materials used in the construction should be low particle shedding and not allow space for contamination/dust to collect that is, be easily accessible to allow for cleaning to occur.
- Cleaning processes should be validated and performed accordingly. The cleanroom should be periodically monitored to ensure that the cleaning protocols are effective and that the environment possesses no risk to the product quality.
- Well qualified and trained staff should be employed to reduce the occurrence of errors.

Quality management system

- All licensed manufacturing facilities should have a quality management system which defines how the requirements of the specific regulatory agencies are enforced to ensure that cell therapy production is within the required legislation. A quality management system will typically include policies around good manufacturing practice noncompliance reporting, validations, materials management, quality assurance procedures, complaint reporting and change control to name but a few.

Cell therapies for limbal stem cell deficiency

- Cells for sight, as a licensed facility, delivers cell-based therapies to patients suffering from blinding eye diseases such as limbal stem cell deficiency ensuring compliance to all of the above. Limbal stem cell deficiency occurs when the cornea, the front surface of the eye, degenerates due to the dysfunction of the limbal stem cell population. Cells for sight have developed an limbal stem cell deficiency therapy consisting of epithelial cells in combination with a novel fibrin gel substrate. The manufacturing process includes stringent procedures and controls in order to ensure the end product is both consistent and of the highest quality.

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Stem cell-based therapy of corneal epithelial and endothelial diseases

Corneal dysfunction is the second leading cause of blindness. Approximately 10 million patients worldwide are affected by some form of corneal disease. More than 50,000 cornea transplants are performed every year, but this procedure is limited by cornea donation availability. Recently, new cell replacement procedures have been developed to treat a variety of corneal diseases. This review will focus on the recent advances in the use of limbal epithelial stem cells (LESCs) to treat corneal epithelial cell deficiency and improvements in replacing dysfunctional corneal endothelial cells (CECs) with exogenous CECs. Several protocols have been developed to differentiate pluripotent stem cells into LESC- or CEC-like cells, potentially yielding an unlimited source for the cell replacement therapy of corneal diseases.

Keywords: cell transplantation • corneal endothelium • corneal transplantation • limbal epithelial stem cells • stem cell differentiation

The eye is a highly specified organ for light and image perception. From the cornea to the retina, every cell type in the eye serves a specific function including light refraction, accommodation, photoelectrical transduction, electrical signal transmission, etc. Thus, degeneration or malfunction of any cell type can lead to different types of ocular diseases. This minireview will focus on the cornea and the various clinical approaches to treat corneal diseases. Readers who are interested in cell therapy of retinal disorders may refer to a recent excellent review paper by Coffey *et al.* [1] that describes the recent progress in transplanting retinal pigment epithelial cells to age-related macular degeneration and Stargardt disease [2].

In humans, the outer surface of the eye sits on a protective structure barrier called the cornea, which is composed of five distinct layers (see Figure 1). The cornea primarily functions to transmit and refract light while keeping the integrity of anterior chamber.

At the outermost surface, corneal epithelium is composed of several layers of corneal epithelial cells that are connected to each

other with tight junctions. Corneal epithelial cells undergo constant self-renewal via basal cell proliferation and differentiation of limbal epithelial stem cells (LESCs) that are found in the corneal limbus located between the transparent cornea and opaque conjunctiva. Any condition that causes the loss or reduction of LESCs to a certain degree will lead to corneal epithelium defect, corneal hazing, or even blindness, which is clinically termed as limbal stem cell deficiency (LSCD). LSCD can be caused by chemical burn or traumatic injury of a large area of cornea surface, hereditary corneal dystrophy and several immune disorders such as Stevens–Johnson syndrome [3].

In contrast to the multiple layers of epithelial cells in the corneal epithelium, the corneal endothelium is comprised of a monolayer of hexagonal endothelial cells. Though a single layer, corneal endothelial cells (CECs) pump extra water to the anterior chamber and regulate the cornea to proper hydration, thus playing a pivotal role in maintaining cornea transparency. CECs also allow small molecules and nutrition to traverse from

Songtao Yuan^{1,2}
& Guoping Fan^{*1}

¹Department of Human Genetics & Broad Stem Cell Research Center, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA 90095, USA

²Department of Ophthalmology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu Province 210029, China

*Author for correspondence:
Tel.: +1 310 267 0439
gfan@mednet.ucla.edu

the aqueous humor to the stromal layer, thus contributing to cellular metabolism of avascular stromal cells [4]. CECs barely proliferate *in vivo*, and the density of CECs decreases with age at the rate of 0.6% per year [5,6]. Upon Fuchs endothelial dystrophy, trauma or a complication of intraocular surgery, etc., which leads to the corneal endothelium's damage, CECs' density decreases below 400–700/mm², and the corneal transparency cannot be maintained due to accumulation of fluid anteriorly into the stroma and epithelium layers. Excess fluid not only clouds the cornea but also forms a blister-like structure between the basal epithelium cells, thus affecting vision and causing pain sensations as described for bullous keratopathy.

The promise of stem-cell-based treatments of corneal diseases

Corneal transplantation (or keratoplasty) is usually the first choice of treatment for many corneal diseases in the clinic. These diseases include corneal leukoma, which affect the patients' visual acuity, bullous keratopathy, advanced keratoconus, etc. Besides penetrating keratoplasty, advanced surgical procedures have also been developed, including limbus transplantation, Descemet's membrane endothelial keratoplasty (DMEK), and deep laminar keratoplasty. However,

hundreds of thousands of patients worldwide are waiting for transplantation surgery due to shortage of corneal donors. Therefore, stem-cell-based treatments have been proposed as a promising way to solve the problem. For example, autologous *ex vivo* expansion of corneal limbal epithelial cells have been used to treat LSCD. In addition, novel methods of using pluripotent stem cells to differentiate to LESC- and CEC-like cells also hold great promise for treating corneal diseases. It is worth noting that we focus on reviewing corneal epithelial and endothelial cell transplantation in this paper and will not discuss the applications of other types of stem cells such as mesenchymal stem cells and corneal stromal stem cells in corneal stroma regeneration [7,8].

Cell sources for treating LSCD

The self-renewal, migration and differentiation of limbal stem cells is essential for maintaining corneal epithelium structural integrity and repairing corneal damage. One of the most recent advances in the treatment of LSCD is autologous cell transplantation after *ex vivo* expansion of LSCs. LSCs are thought to be precursors of corneal epithelial cells [9], and was one of earliest stem cells applied on clinical applications [10–12]. The concept of LSCs was not clear in the early clinical application, and the property of transplanted stem cells were hotly debated [13]. However, it is now well accepted that LSCs are present in the limbal biopsy. Furthermore, LSC morphology and putative markers are now routinely used to identify LSCs, such as small cell size, high nucleus/cytoplasm ratio and euchromatin rich nuclei [14,15].

Early efforts focused on the identification and validation of LSC markers including OCT4, LGR5, integrins ($\alpha 9$ and $\beta 1$), NGF receptors (TrkA), CK15, CK19, etc. [16,17]. Ultimately, ABCG2, Δ Np63 α , C/EBP δ and Bmi-1 are now accepted as putative LSC markers.

ABCG2 is a member of the ATP binding cassette transporters and recognized as a universal marker of stem cells [18–20]. In addition, expression of ABCG2 was also found in a number of cancer cells and appears to also be a marker of cancer stem cells [21,22]. Δ Np63 α is a truncated transcriptional variant of the p63 gene, which has six isoforms. High p63 content is present in limbal epithelial cells and suggested as a putative LSC marker [9]. Δ Np63 β and γ isoforms were regarded as promoters to epithelial cell differentiation, and Δ Np63 α is accepted as another marker of LSCs [23–25]. Bararo *et al.* [24] showed that coexpression of C/EBP δ , Bmi1 and Δ Np63 α can be used to identify resting limbal stem cells. C/EBP δ , but not Δ Np63 α , indefinitely promotes holoclone self-renewal and prevents clonal evolution. Recently, it was shown



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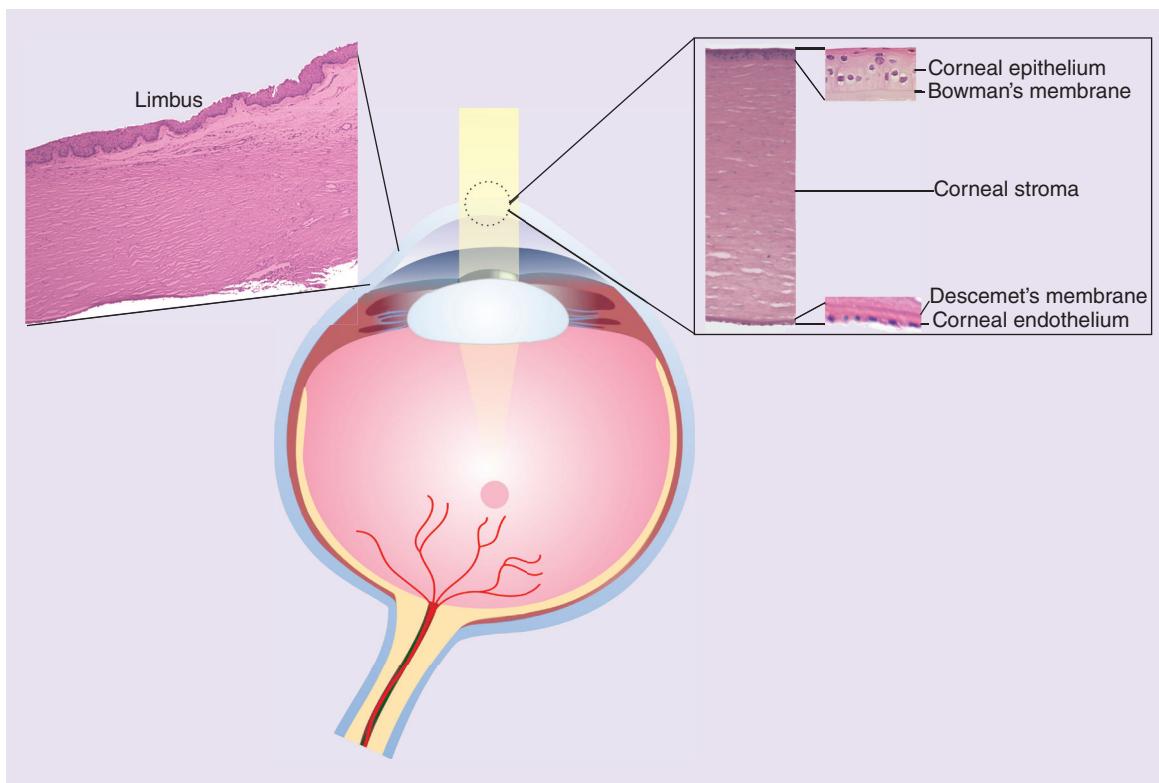


Figure 1. The cornea structure in the eye. The inset illustrates five layers of cornea including corneal epithelium, Bowman's membrane, stromal layer, Descemet's membrane and a monolayer of corneal endothelial cells .

that ABCB5 is a substantial marker for LESC [26]. ABCB5 was found to coexpress with p63 α in human LESC and play a pivotal role in corneal epithelium development and requirement. All of these proteins are not highly specific markers for LESC; many different stem cells express some of these markers. Therefore, these markers are often used in combination to identify bona-fide LESC.

Autologous LESC is an ideal source of corneal epithelial transplantation due to the favorable lack of immune rejection. But, indications for autologous LESC transplantation are limited. The procedure requires some healthy limbal tissue. Therefore diseases such as in Steven-Johnson Syndrome and other diseases, which may cause extensive damage of eye surface bilaterally, cannot be treated by autologous LESC. Thus, other cell sources are required to treat these difficult cases of LSCL. For example, LESC from close relatives of patients are a sensible source for transplantation, with the only disadvantage of potential graft rejection. Because LESC are the obvious target for stem cell differentiation, a wide range of stem cell sources are tested for differentiation into LESC-like cells. Somatic stem cells like bone-marrow-derived mesenchymal stem cells [27,28], hair follicle stem cells [29,30], dental pulp stem cells [31], umbilical cord stem cells [32] and skin epithelial stem cells [33] have been used to reconstruct

the cornel epithelium. Most recently, two studies carefully examined homeostasis of limbal stem cells and found that the Wnt7A-Pax6 axis is required for the development and maintenance of limbal stem cells [33]. Furthermore, one group showed that ABCB5 is a major marker labeling limbal stem cells in both human and murine limbal stem cells [80].

Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) show no obvious advantage in differentiation and transplantation to corneal epithelium compared with somatic stem cells. However, ESCs and iPSCs could be mass-produced due to their unlimited proliferation capacity. Moreover, iPSCs can be considered autologous, which is thought to reduce the immune reaction. Homma *et al.* first reported the differentiation of ESCs to epithelial progenitor cells and the reconstruction of mice corneal surface [34]. Subsequently, ESCs were found to be capable of differentiating into a monolayer of epithelium-like cells [35,36]. iPSCs also showed a similar differentiation potential 1 [37,38]. Recently, researchers found that proper limbal niche, including specialized extracellular matrix and cytokines, is essential for maintaining LESC and differentiation of corneal epithelial cells [39]. Ahmad *et al.* showed that human ESCs differentiate into corneal epithelial-like cells on collagen IV using medium conditioned by the limbal fibroblasts [36]. A variety of cell sources for treat-

ing LSCD are summarized in **Table 1**, including LESCs, somatic stem cells, and pluripotent stem-cell-derived corneal epithelium lineage cells.

Cell sources for corneal endothelial diseases

Compared to corneal epithelium, the protocol for *ex vivo* expansion of autologous CECs is not well established yet. The main reason is due to limited CECs' proliferative capacity *in vitro* [40–42], which also results in the smaller number of CECs studies compared with corneal epithelial cells. It is estimated that the adult primary CECs can be passaged around four times, and there is little improvement even with modified medium and supplemented cytokines [43]. Yet, several protocols are developed to promote the proliferation of CECs, including the use of human bone marrow mesenchymal stem-cell-derived conditioned medium [44], or human amniotic epithelial-cell-derived conditional

medium [45] (**Table 2**). In addition, telomerase or *Cdk4R24C* (constitutively active mutant form of *Cdk4*) and *CyclinD1* transduction into CECs showed *in vitro* pump function [46,47]. Although no oncogenes were transduced, clinical safety is still a concern for these genetically modified cell lines. Hirata-Tominaga *et al.* recently studied the important role for LGR5 in maintaining the fate of CECs, and they found that the ligand RSPO-1 could stimulate CECs proliferation *in vitro* [48]. Gao *et al.* developed a protocol for fetal CEC culture; however, the authors found that fetal CECs do not exhibit a higher proliferative capacity [49].

Another source for CEC transplantation is corneal precursors (**Table 2**). Both corneal stromal and endothelial cells contain a significant number of precursors. Yoshida *et al.* reported isolation of cornea-derived precursors from the mouse corneal stroma which has characteristics of multipotent neural crest-derived stem

Table 1. Sources and procedures of limbal epithelial stem cell transplantation to treat limbal stem cell deficiency.

Cell sources	Procedure	Result and highlight	Ref.
Clinical practices			
Human LESCs	Autologous and allogenic LESCs transplantation	Multiple LSCDs were treated. Structural and functional improvement in long-term follow-up reports.	[10–12,65–71]
Human conjunctival epithelial cells	Autologous transplantation	<i>Ex vivo</i> procedure with certain therapeutic effect, but long-term evaluation is needed.	[73]
Human oral mucosal epithelial cell	Autologous transplantation	<i>Ex vivo</i> procedure, and visual acuity improvement in a majority of patients.	[74,75]
Animal experiments			
Human and rat mesenchymal stem cells	Differentiated to corneal epithelial cells, and transplantation	Showed therapeutic effects on rat model, but the effect may be associated with the inhibition of inflammation by MSCs.	[27,28]
Mouse hair follicle stem cells	Transdifferentiated to corneal epithelia like cells, and transplantation	The ocular surface was reconstructed on mice model.	[29,30]
Human dental pulp stem cells	Seeded on amniotic membrane for transplantation	Improved corneal transparency and reconstructed corneal epithelium but unclear about the mechanism of therapeutic effect.	[31]
Human umbilical cord lining stem cells	Seeded on amniotic membrane for allogenic transplantation	Express some LESCs markers, and showed the therapeutic effect on a rabbit injury model.	[32]
Skin epithelial stem cells	Transdifferentiation of skin epithelial stem cells to ESC and transplantation	Defined the signal pathway and transcription factor that keep the corneal epithelial fate and proved the function of ESC-like cells transduced with Pax-6.	[33]
Mouse ESCs derived corneal epithelial like cells	Dissociated epithelial progenitors from mESCs transplantation	mESCs were induced to corneal epithelial progenitors. Corneal epithelium recovery within 24 h after transplantation.	[34,35]

ESC: Embryonic stem cell; LESCs: Limbal epithelial stem cell; LSCD: Limbal stem cell deficiency; mESC: mouse Embryonic Stem Cell; MSC: Mesenchymal Stem Cells.

Table 2. Cell sources for corneal endothelial cell transplantation on animal models.

Cell sources	Procedure	Result and highlight	Ref.
Adult human CECs	<i>In vitro</i> culture	Showed limited <i>in vitro</i> proliferative capacity of adult CEC.	[40–45]
	Establishment of CEC cell lines	CEC lines were generated by transduction of telomerase or <i>Cdk4R24C</i> (constitutively active mutant form of <i>Cdk4</i>) and <i>CyclinD1</i> gene.	[46,47]
Fetal human CEC	Fetal CEC culture	Protocol of primary culture, passage and freezing fetal CECs.	[49]
Human and mice corneal precursors	Corneal stromal stem cells <i>in vitro</i> culture, differentiation and transplantation	There are precursors in corneal stroma, which could be induced to CEC-like cells, and showed function on rabbit model.	[50–52]
	Culture of stem cells from human corneal endothelium.	There are precursors in corneal endothelium.	[53]
Human ESCs	Human ESCs differentiated to CECs and transplantation	ESCs were differentiated to CECs and showed function on rabbit corneal endothelium damage model for the first time.	[56]
Multi sources of multipotent stem cells	Rat neural crest cells, human umbilical cord blood mesenchymal stem cells, human fetal bone-marrow-derived endothelial progenitor cell differentiation.	These cells were induced to CEC-like cells and showed CECs' character <i>in vitro</i> .	[57–60]

CEC: Corneal endothelial cell; ESC: Embryonic stem cell.

cells [50,51]. A procedure where CECs were differentiated from mouse corneal stromal precursors by retinoic acid and activation of Wnt/(beta)catenin signaling was also reported, which confirmed the function of these CECs on a rabbit corneal disease model, and human CEC-like cells were acquired from differentiation of human corneal stromal precursors [52]. Corneal endothelial precursors were also found by sphere-forming assay [53] (**Table 2**). It is suggested that corneal endothelial stem cells may reside within the periphery of corneal endothelium and continually migrate centripetally from the extreme periphery to the center of the cornea endothelium [54]. It seems that the peripheral cell populations have a higher density of precursors than the central part of the corneal endothelium [55].

Besides cell sources from cornea tissue, other stem cells, such as ESCs [56], cord blood mesenchymal stem cells [57], fetal bone-marrow-derived endothelial progenitor cells [58], adipose-derived stem cells [59] and neural crest cells [60] were differentiated to corneal endothelial-like cells. But iPSC-derived CEC-like cells have not been reported yet. Conditional medium or coculture system were applied in these stem cell differentiation protocols, meaning the specific molecule or signal pathway for CEC differentiation is still unclear. But, it looks like that CECs' differentiation need interact with signals from other type of cells at the anterior eye segment [61], because not only CECs but also lens epithelial cells and CEC's biomimetic environment were utilized to drive these stem cells to CEC-like cells.

One of the crucial criteria to obtain CECs *in vitro* is to characterize cells with authentic markers of CECs. Although ZO-1, Na⁺-K⁺-ATPase and Occludin are used as the putative markers for CECs, they are also expressed by many other tissues, such as retinal pigment epithelial cells. Therefore, identification of additional specific markers for CECs is important to properly characterize the differentiation of CECs. Recently, our lab analyzed mRNA transcriptome in human fetal and adult CECs, and identified novel markers including Wnt5a, S100A4, S100A6 and IER3 as additional specific CEC markers in either fetal or adult stages [62]. In addition, Glycican-4 and CD200 were reported to distinguish human corneal endothelium from stromal fibroblasts [63]. The availability of these new markers would be helpful to characterize CEC-like cells derived from ESCs and iPSCs [64].

Surgical procedures & therapeutic effects

The derivation of specific subtypes of corneal cells from stem cells is only the first step toward the treatment of corneal diseases. Indeed, it is equally important to develop good clinical procedures for delivering cells into corneal tissue. Below we review the recent progresses on the surgical procedures to deliver LESCs or CECs to treat LSCD and CEC deficiency.

Clinical application of LESCs

The clinical application of LESCs has a long history, especially for autologous LESC transplantation [10], which is now widely accepted to be the top

choice for treating LSCD [65–70]. For autologous LESCs transplantation, patient's contralateral eye's LESCs were cultured on fibrin or amniotic membrane, then transplanted to the eye with LCSD (Table 1). Surgically, a small biopsy from patient's contralateral eye is easy and safe, which can be operated in an outpatient clinic. In certain cases when autologous LESCs are not available from patients themselves, a biopsy of limbus from relatives or donor eye is an alternative to allow *ex vivo* expansion of LESCs. Because cultured cells do not have antigen presenting cells, transplantation of *ex vivo* expanded LESCs exhibits lower rejection rate compared with direct limbal transplantation. Finally, transplantation of *ex vivo* expansion of LESCs has demonstrated the best satisfactory therapeutic effect on LSCD with minimal trauma to contralateral eye.

Two clinical studies have reported the outcome of LESCs transplantation in more than several hundred patients over the period of a decade or longer [68,70]. According to these reports, over 70% patients' corneal surfaces were functionally restored and kept stable, and some patients have been followed up over 10 years. Rama *et al.* reported that cultures containing more than 3.0% of Δ Np63 α + (an LESCs marker) holoclones were successful in almost 80% of patients. If cultures contained 3.0% or less of Δ Np63 α + cells, the success rate drops to 11%. Rama *et al.* reported a way to improve the surgery success rate of LESCs transplantation by enriching the p63+ cells in *ex vivo* culture. Other factors affecting the prognosis of LESCs transplantation include severe tear film deficiency, uncontrolled inflammation and adnexal abnormalities [71,72].

In clinical practice, LSCD is also treated with other epithelial cells such as conjunctiva epithelium [73] and oral mucosal epithelium [74,75]. Some encouraging results were obtained from these techniques, but the number of clinical treatments is still very small and no superior clinical outcome was demonstrated when compared with the LESCs transplantation. However, these two types of epithelia are easily acquired and can be applied to treat bilateral LSCD. Additionally, many other types of somatic stem cells are tested in clinical trial, or in preclinical animal models, including bone-marrow-derived mesenchymal stem cells [27,28], hair follicle stem cells [29], dental pulp stem cells [31], umbilical cord stem cells [32] and skin stem cells [33]. Finally, the potential use of pluripotent stem-cell-derived LESCs-like cells are still at the stage of preclinical studies, awaiting for testing in small and large animal models of LSCD. With deepened understanding on LESCs's differentiation, more stem cell sources will available in clinical application in future. The indication for each type of source needs further research to identify.

CEC transplantation procedures

Due to the lack of donor eye, new procedures were developed for transplantations of CECs. Recently, Descemet's stripping endothelial keratoplasty and DMEK [76] procedures showed better visual acuity improvement in clinical practice. But, there is no clinical report on *ex vivo* CEC treatment like LESCs, and cadavers are the only source for CECs transplantation. Because CECs have limited proliferative capacity *in vitro*, so the limited CEC source is still the major obstacle for CEC transplantation. Recently, some exciting progresses were reported in animal model experiments. CEC-like cells from ESCs were transplanted to rabbit CEC dysfunction model and showed therapeutic effect [56]. Mimura *et al.* published a rabbit model of CEC transplantation with cultured human CECs or CEC precursor cells [77,78]. Human CECs or CEC precursors were expanded *ex vivo* on collagen sheet, which were then transplanted into rabbit eyes that were stripped off CECs. After 3–4 weeks, they observed excellent therapeutic effects on corneal transparency in CEC transplanted eyes. The same strategy was applied in the monkey CEC deficiency model [79], with the monkey corneal edema showing recovery in clarity and decrease in overall corneal thickness. CEC-like cells derived from human umbilical cord blood mesenchymal stem cells [57], fetal bone-marrow-derived endothelial progenitor cells [58] or neural crest cells [60] were also tested, and exhibited modest therapeutic effect.

Although the above preclinical experiments indicate a promising strategy for clinical application, several concerns remain to be addressed, such as the safety of stem cells, therapeutic effect of the new procedure compared with DMEK and Descemet's stripping endothelial keratoplasty with donated cornea. Meanwhile, the limited proliferative capacity of primary CECs is still a rate-limiting factor for *ex vivo* expansion, so the clinical potential is still uncertain. Nevertheless, because CEC transplantation is a relative immune-privileged site for corneal transplantation, if CEC-like cells from ESCs or iPSCs are successfully developed, CEC replacement therapy with sheet transplantation would be of great value in the treatment of CEC deficiency.

Conclusion

For eye diseases due to the deficiency of LESCs, autologous and allogenic limbal stem cells have been successfully used to treat LSCD patients in the past decade. Unilateral LSCD can be effectively treated by transplantation of autologous LESCs via *ex vivo* expansion. However, this procedure needs an biopsy from patients's contralateral healthy eye, thus posing a potential risk for the healthy eye. For patients with

bilateral LSCD, allograft LESC transplantation is the option, but patients may face graft rejection in the long run. Therefore, LESCs derived from hESCs and hiPSCs would be very useful for clinical treatment of either unilateral or bilateral LSCD. At present, the efficacy and safety in the treatment of LSCD with mucosal and conjunctival epithelial cells remain to be proven by long-term follow-up of a large cohort of patients. In a parallel situation, patients with CEC diseases can be treated via transplantation of CEC sheet from donor eyes. Because pluripotent stem cells can be induced into functional CECs or CEC-like cells *in vitro*, we expect that stem-cell-derived CECs would be available for treating CEC deficiency in the near future.

Future perspective

With increased understanding of the molecular events underlying corneal epithelial and endothelial lineage differentiation, pluripotent and somatic stem cells would be effectively induced to differentiate into LESCs and CECs. Future clinical trials would also determine the concern of the immune rejection, the efficacy and safety of either hESC- or hiPSC-derived LESCs and CECs *in vivo*. Considering the advantage of manufacturing a large quantity of clinical-

grade hESCs or hiPSCs for cell differentiation, we believe that a bank of human ESC- and iPSC-derived LESCs would provide a most useful and economic cell source for treating LSCD patients who cannot pursue the *ex vivo* expansion of autologous LESCs. Finally, although stem-cell-derived LESC and CECs have been tested for the efficacy and safety in animal models of LSCD and CEC deficiency, only rigorous clinical trials and long-term follow-up of patients would eventually vindicate stem-cell-based therapy for treating patients with corneal epithelial and endothelial diseases.

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Executive summary

Patients with corneal epithelial & endothelial diseases require stem-cell-based therapy

- Corneal transplantation is a cure to many severe corneal diseases. However, the donor shortage in developing countries greatly hindered the treatment of corneal diseases worldwide.
- Limbal stem cell deficiency (LSCD) is a group of diseases with defects in limbal epithelial stem cells (LESCs) that can be treated by transplantation of either *ex vivo* expansion of LESCs or stem-cell-derived LESCs.
- Due to the paucity of proliferation capacity of adult corneal endothelial cells (CECs) *ex vivo*, CEC density decreases with age. CEC deficiency can be caused by degenerative conditions, trauma and intraocular surgery procedure.

Regenerative medicine for the treatment of LSCD & CEC deficiency

- Autologous *ex vivo* expansion of LESCs and transplantation is the first choice for unilateral LSCD with a successful long-term follow-up record.
- LESCs derived from a variety of somatic and pluripotent stem cells are promising for the treatment of both unilateral and bilateral LSCD patients.
- Current treatment of CEC deficiency is limited to CEC sheet transplantation or cornea transplantation.
- Limited success is achieved in the differentiation of stem cells into CECs.
- With the improvement of CEC transplantation procedure such as Descemet's membrane endothelial keratoplasty, stem-cell-derived CEC transplantation holds a great promise for treating CEC deficiency diseases in the near future.

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