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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

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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

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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/mm^2, 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 LESCs. LESCs 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 LESCs 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 LESCs are present in the limbal biopsy. Furthermore, LESC morphology and putative markers are now routinely used to identify LESCs, such as small cell size, high nucleus/cytoplasm ratio and euchromatin rich nuclei [14,15].

Early efforts focused on the identification and validation of LESC markers including OCT4, LGR5, integrins (α9 and β1), NGF receptors (TrkA), CK15, CK19, etc. [16,17]. Ultimately, ABCG2, ΔNp63α, C/EBPδ and Bmi-1 are now accepted as putative LESC 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]. ΔNp63α 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 LESC marker [9]. ΔNp63 β and γ isoforms were regarded as promoters to epithelial cell differentiation, and ΔNp63α is accepted as another marker of LESCs [23–25]. Bararo *et al.* [24] showed that coexpression of C/EBPδ, Bmi1 and ΔNp63α can be used to identify resting limbal stem cells. C/EBPδ, but not ΔNp63α, indefinitely promotes holoclone self-renewal and prevents clonal evolution. Recently, it was shown

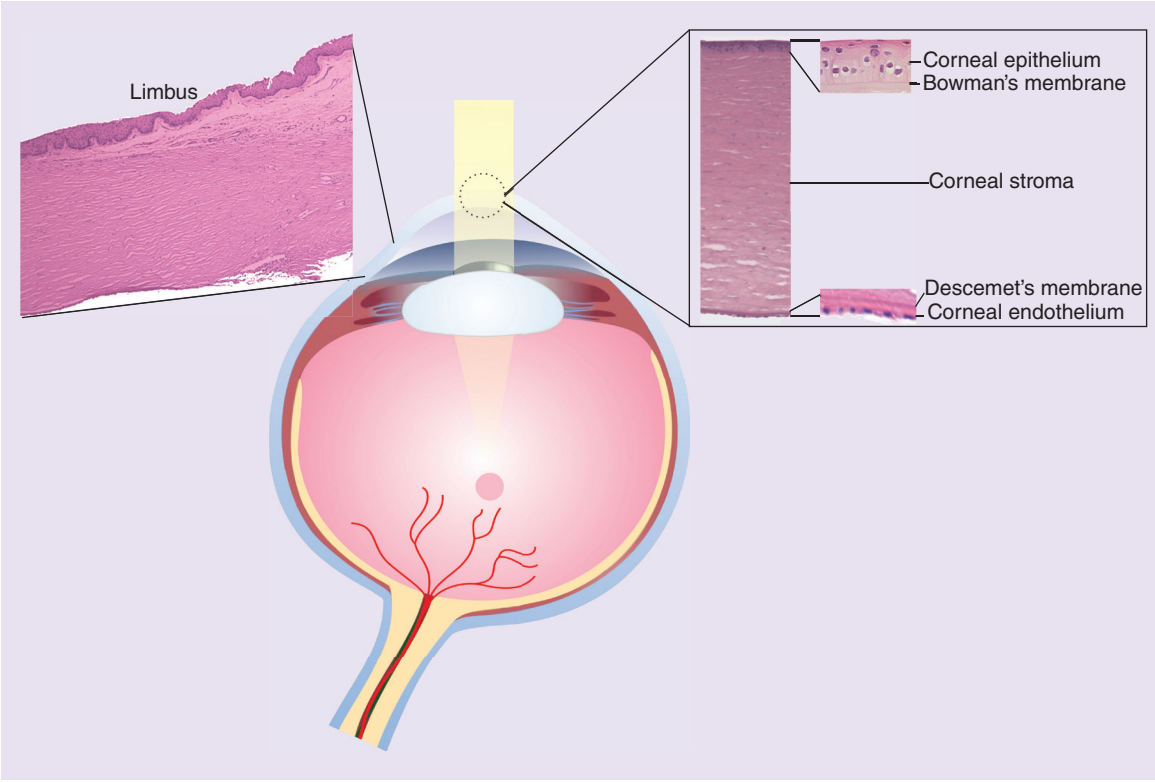

Figure 1. The cornea structure in the eye. The inlet illustrates five layers of cornea including corneal epithelium, Bowman's membrane, stromal layer, Descemet's membrane and a monolayer of corneal endothelial cells .

that ABCB5 is a substantial marker for LESCs [26]. ABCB5 was found to coexpress with p63α in human LESCs and play a pivotal role in corneal epithelium development and requirement. All of these proteins are not highly specific markers for LESCs; many different stem cells express some of these markers. Therefore, these markers are often used in combination to identify bona-fide LESCs.

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 LESCs. Thus, other cell sources are required to treat these difficult cases of LSCD. For example, LESCs from close relatives of patients are a sensible source for transplantation, with the only disadvantage of potential graft rejection. Because LESCs 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 l [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	*Ex vivo* procedure with certain therapeutic effect, but long-term evaluation is needed.	[73]
Human oral mucosal epithelial cell	Autologous transplantation	*Ex vivo* 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 LESC and transplantation	Defined the signal pathway and transcription factor that keep the corneal epithelial fate and proved the function of LESC-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; LESC: 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	*In vitro* culture	Showed limited *in vitro* proliferative capacity of adult CEC.	[40–45]
	Establishment of CEC cell lines	CEC lines were generated by transduction of telomerase or *Cdk4R24C* (constitutively active mutant form of Cdk4) and *CyclinD1* 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 *in vitro* 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 *in vitro*.	[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 centripetically 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, Glypican-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 LESC 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 LESC has demonstrated the best satisfactory therapeutic effect on LSCD with minimal trauma to contralateral eye.

Two clinical studies have reported the outcome of LESC 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 ΔNp63α+ (an LESC marker) holoclones were successful in almost 80% of patients. If cultures contained 3.0% or less of ΔNp63α+ cells, the success rate drops to 11%. Rama *et al.* reported a way to improve the surgery success rate of LESC transplantation by enriching the p63+ cells in *ex vivo* culture. Other factors affecting the prognosis of LESC 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 LESC transplantation. However, these two types of epithelia are easily acquired and can be applied to treat bilateral LCSD. 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 LESC-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 LESC'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 linage 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 hiSPC-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.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 Ramsden CM, Powner MB, Carr AJ, Smart MJ, Da Cruz L, Coffey PJ. *Stem Cells* in retinal regeneration: past, present and future. *Development* 140(12), 2576–2585 (2013).

2 Schwartz SD, Hubschman JP, Heilwell G *et al.* Embryonic stem cell trials for macular degeneration: a preliminary report. *Lancet* 379(9817), 713–720 (2012).

3 Wright B. *Corneal Regenerative Medicine: Methods And Protocols.* Springer, London, UK, 8 (2013).

4 Waring GO 3rd, Bourne WM, Edelhauser HF, Kenyon KR. The corneal endothelium. Normal and pathologic structure and function. *Ophthalmology* 89(6), 531–590 (1982).

5 Bourne WM, Nelson LR, Hodge DO. Central corneal endothelial cell changes over a ten-year period. *Invest. Ophthalmol. Vis. Sci.* 38(3), 779–782 (1997).

6 Joyce NC. Proliferative capacity of the corneal endothelium. *Prog. Retin. Eye Res.* 22(3), 359–389 (2003).

7 Hirayama M, Oshima M, Tsuji T. Development and prospects of organ replacement regenerative therapy. *Cornea* 32(Suppl. 1), S13–S21 (2013).

8 Wu J, Rnjak-Kovacina J, Du Y, Funderburgh ML, Kaplan DL, Funderburgh JL. Corneal stromal bioequivalents secreted on patterned silk substrates. *Biomaterials* 35(12), 3744–3755 (2014).

9 Pellegrini G, Dellambra E, Golisano O *et al.* p63 identifies keratinocyte stem cells. *Proc. Natl Acad. Sci. USA* 98(6), 3156–3161 (2001).

10 Kenyon KR, Tseng SC. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology* 96(5), 709–722; discussion 722–703 (1989).

11 Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet* 349(9057), 990–993 (1997).

•• **The limbal epithelial stem cells *ex vivo* expansion and clinical application were reported for the first time, which promote the establishment of limbal epithelial stem cells' concept.**

12 Tsubota K, Satake Y, Kaido M *et al.* Treatment of severe ocular-surface disorders with corneal epithelial stem-cell transplantation. *N. Engl. J. Med.* 340(22), 1697–1703 (1999).

13 Tsubota K. Corneal epithelial stem-cell transplantation. *Lancet* 349(9064), 1556 (1997).

14 Romano AC, Espana EM, Yoo SH, Budak MT, Wolosin JM, Tseng SC. Different cell sizes in human limbal and central corneal basal epithelia measured by confocal microscopy and flow cytometry. *Invest. Ophthalmol. Vis. Sci.* 44(12), 5125–5129 (2003).

15 Chen Z, De Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells* 22(3), 355–366 (2004).

16 Pellegrini G, Rama P, Mavilio F, De Luca M. Epithelial *Stem Cells* in corneal regeneration and epidermal gene therapy. *J. Pathol.* 217(2), 217–228 (2009).

17 Takacs L, Toth E, Berta A, Vereb G. *Stem Cells* of the adult cornea: from cytometric markers to therapeutic applications. *Cytometry A* 75(1), 54–66 (2009).

18 Zhou S, Schuetz JD, Bunting KD *et al.* The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of *Stem Cells* and is a molecular determinant of the side-population phenotype. *Nat. Med.* 7(9), 1028–1034 (2001).

19 Scharenberg CW, Harkey MA, Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 99(2), 507–512 (2002).

20 Watanabe K, Nishida K, Yamato M *et al.* Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. *FEBS Lett.* 565(1–3), 6–10 (2004).

21 Zen Y, Fujii T, Yoshikawa S *et al.* Histological and culture studies with respect to ABCG2 expression support the existence of a cancer cell hierarchy in human hepatocellular carcinoma. *Am. J. Pathol.* 170(5), 1750–1762 (2007).

22 Diestra JE, Scheffer GL, Catala I *et al.* Frequent expression of the multi-drug resistance-associated protein BCRP/MXR/ABCP/ABCG2 in human tumours detected by the BXP-21 monoclonal antibody in paraffin-embedded material. *J. Pathol.* 198(2), 213–219 (2002).

23 Wang DY, Cheng CC, Kao MH, Hsueh YJ, Ma DH, Chen JK. Regulation of limbal keratinocyte proliferation and differentiation by TAp63 and DeltaNp63 transcription factors. *Invest. Ophthalmol. Vis. Sci.* 46(9), 3102–3108 (2005).

24 Barbaro V, Testa A, Di Iorio E, Mavilio F, Pellegrini G, De Luca M. C/EBPdelta regulates cell cycle and self-renewal of human limbal stem cells. *J. Cell Biol.* 177(6), 1037–1049 (2007).

25 Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De Luca M. Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proc. Natl Acad. Sci. USA* 102(27), 9523–9528 (2005).

26 Ksander BR, Kolovou PE, Wilson BJ *et al.* ABCB5 is a limbal stem cell gene required for corneal development and repair. *Nature* 511(7509), 353–357 (2014).

• **Identified a marker for limbal epithelial stem cells, but also clarified the ABCB5's pivotal role in keeping the stemness of limbal epithelial stem cells.**

27 Ma Y, Xu Y, Xiao Z *et al.* Reconstruction of chemically burned rat corneal surface by bone marrow-derived human mesenchymal stem cells. *Stem Cells* 24(2), 315–321 (2006).

28 Jiang TS, Cai L, Ji WY *et al.* Reconstruction of the corneal epithelium with induced marrow mesenchymal *Stem Cells* in rats. *Mol. Vis.* 16, 1304–1316 (2010).

29 Blazejewska EA, Schlotzer-Schrehardt U, Zenkel M *et al.* Corneal limbal microenvironment can induce transdifferentiation of hair follicle *Stem Cells* into corneal epithelial-like cells. *Stem Cells* 27(3), 642–652 (2009).

30 Meyer-Blazejewska EA, Call MK, Yamanaka O *et al.* From hair to cornea: toward the therapeutic use of hair follicle-derived *Stem Cells* in the treatment of limbal stem cell deficiency. *Stem Cells* 29(1), 57–66 (2011).

31 Gomes JA, Geraldes Monteiro B, Melo GB *et al.* Corneal reconstruction with tissue-engineered cell sheets composed of human immature dental pulp stem cells. *Invest. Ophthalmol. Vis. Sci.* 51(3), 1408–1414 (2010).

32 Reza HM, Ng BY, Gimeno FL, Phan TT, Ang LP. Umbilical cord lining *Stem Cells* as a novel and promising source for ocular surface regeneration. *Stem Cell Rev.* 7(4), 935–947 (2011).

33 Ouyang H, Xue Y, Lin Y *et al.* WNT7A and PAX6 define corneal epithelium homeostasis and pathogenesis. *Nature* 511(7509), 358–361 (2014).

•• **Identified the transcriptional factor that determin the limbal epithelial stem cells' (LESCs') fate. Transduced skin *Stem Cells* were trans-differentiated to LESCs, which provieded another cell scource for LESCs.**

34 Homma R, Yoshikawa H, Takeno M *et al.* Induction of epithelial progenitors *in vitro* from mouse embryonic *Stem*

Cells and application for reconstruction of damaged cornea in mice. *Invest. Ophthalmol. Vis. Sci.* 45(12), 4320–4326 (2004).

35 Ueno H, Kurokawa MS, Kayama M *et al.* Experimental transplantation of corneal epithelium-like cells induced by Pax6 gene transfection of mouse embryonic stem cells. *Cornea* 26(10), 1220–1227 (2007).

36 Ahmad S, Stewart R, Yung S *et al.* Differentiation of human embryonic *Stem Cells* into corneal epithelial-like cells by *in vitro* replication of the corneal epithelial stem cell niche. *Stem Cells* 25(5), 1145–1155 (2007).

37 Hayashi R, Ishikawa Y, Ito M *et al.* Generation of corneal epithelial cells from induced pluripotent *Stem Cells* derived from human dermal fibroblast and corneal limbal epithelium. *PLoS ONE* 7(9), e45435 (2012).

38 Shalom-Feuerstein R, Serror L, De La Forest Divonne S *et al.* Pluripotent stem cell model reveals essential roles for miR-450b-5p and miR-184 in embryonic corneal lineage specification. *Stem Cells* 30(5), 898–909 (2012).

39 Li W, Hayashida Y, Chen YT, Tseng SC. Niche regulation of corneal epithelial *Stem Cells* at the limbus. *Cell Res.* 17(1), 26–36 (2007).

40 Bohnke M, Eggli P, Engelmann K. Transplantation of cultured adult human or porcine corneal endothelial cells onto human recipients *in vitro.* Part II: Evaluation in the scanning electron microscope. *Cornea* 18(2), 207–213 (1999).

41 Amano S. Transplantation of cultured human corneal endothelial cells. *Cornea* 22(7 Suppl.), S66–S74 (2003).

42 Pistsov MY, Sadovnikova E, Danilov SM. Human corneal endothelial cells: isolation, characterization and long-term cultivation. *Exp. Eye Res.* 47(3), 403–414 (1988).

43 Joyce NC, Zhu CC. Human corneal endothelial cell proliferation: potential for use in regenerative medicine. *Cornea* 23(8 Suppl.), S8–S19 (2004).

44 Nakahara M, Okumura N, Kay EP *et al.* Corneal endothelial expansion promoted by human bone marrow mesenchymal stem cell-derived conditioned medium. *PLoS ONE* 8(7), e69009 (2013).

45 Sha X, Liu Z, Song L, Wang Z, Liang X. Human amniotic epithelial cell niche enhances the functional properties of human corneal endothelial cells via inhibiting P53-survivin-mitochondria axis. *Exp. Eye Res. 116C*, 36–46 (2013).

46 Yokoi T, Seko Y, Yokoi T *et al.* Establishment of functioning human corneal endothelial cell line with high growth potential. *PLoS ONE* 7(1), e29677 (2012).

47 Schmedt T, Chen Y, Nguyen TT, Li S, Bonanno JA, Jurkunas UV. Telomerase immortalization of human corneal endothelial cells yields functional hexagonal monolayers. *PLoS ONE* 7(12), e51427 (2012).

48 Hirata-Tominaga K, Nakamura T, Okumura N *et al.* Corneal endothelial cell fate is maintained by LGR5 through the regulation of hedgehog and Wnt pathway. *Stem Cells* 31(7), 1396–1407 (2013).

49 Gao Y, Zhou Q, Qu M, Yang L, Wang Y, Shi W. *In vitro* culture of human fetal corneal endothelial cells. *Graefes Arch. Clin. Exp. Ophthalmol.* 249(5), 663–669 (2011).

50 Yoshida S, Shimmura S, Shimazaki J, Shinozaki N, Tsubota K. Serum-free spheroid culture of mouse corneal keratocytes. *Invest. Ophthalmol. Vis. Sci.* 46(5), 1653–1658 (2005).

51 Yoshida S, Shimmura S, Nagoshi N *et al.* Isolation of multipotent neural crest-derived *Stem Cells* from the adult mouse cornea. *Stem Cells* 24(12), 2714–2722 (2006).

52 Hatou S, Yoshida S, Higa K *et al.* Functional corneal endothelium derived from corneal stroma *Stem Cells* of neural crest origin by retinoic acid and Wnt/beta-catenin signaling. *Stem Cells Dev.* 22(5), 828–839 (2013).

53 Mimura T, Yamagami S, Yokoo S, Usui T, Amano S. Selective isolation of young cells from human corneal endothelium by the sphere-forming assay. *Tissue Eng. C Methods* 16(4), 803–812 (2010).

54 He Z, Campolmi N, Gain P *et al.* Revisited microanatomy of the corneal endothelial periphery: new evidence for continuous centripetal migration of endothelial cells in humans. *Stem Cells* 30(11), 2523–2534 (2012).

55 Yamagami S, Yokoo S, Mimura T, Takato T, Araie M, Amano S. Distribution of precursors in human corneal stromal cells and endothelial cells. *Ophthalmology* 114(3), 433–439 (2007).

56 Zhang K, Pang K, Wu X. Isolation and Transplantation of Corneal Endothelial Cell-Like Cells Derived from In-Vitro-Differentiated Human Embryonic Stem Cells. *Stem Cells Dev.* 23(12), 1340–1354 (2014).

• **This is the first paper about human pluripotent *Stem Cells* derived corneal endothelial cell like cells that showed *in vivo* function in rabbit experiments.**

57 Joyce NC, Harris DL, Markov V, Zhang Z, Saitta B. Potential of human umbilical cord blood mesenchymal *Stem Cells* to heal damaged corneal endothelium. *Mol. Vis.* 18, 547–564 (2012).

58 Shao C, Fu Y, Lu W, Fan X. Bone marrow-derived endothelial progenitor cells: a promising therapeutic alternative for corneal endothelial dysfunction. *Cells, tissues, organs* 193(4), 253–263 (2011).

59 Dai Y, Guo Y, Wang C *et al.* Non-genetic direct reprogramming and biomimetic platforms in a preliminary study for adipose-derived *Stem Cells* into corneal endothelia-like cells. *PLoS ONE* 9(10), e109856 (2014).

60 Ju C, Zhang K, Wu X. Derivation of corneal endothelial cell-like cells from rat neural crest cells *in vitro*. *PloS ONE* 7(7), e42378 (2012).

61 Silla ZT, Naidoo J, Kidson SH, Sommer P. Signals from the lens and Foxc1 regulate the expression of key genes during the onset of corneal endothelial development. *Exp. Cell Res.* 322(2), 381–388 (2014).

62 Chen Y, Huang K, Nakatsu MN, Xue Z, Deng SX, Fan G. Identification of novel molecular markers through transcriptomic analysis in human fetal and adult corneal endothelial cells. *Hum. Mol. Genet.* 22(7), 1271–1279 (2013).

• **Transciptome analysis on corneal endothelial cells (CECs) provided vauable imformation on CEC's development and identified the CECs' novel functional markers.**

63 Cheong YK, Ngoh ZX, Peh GS *et al.* Identification of cell surface markers glypican-4 and CD200 that differentiate human corneal endothelium from stromal fibroblasts. *Invest. Ophthalmol. Vis. Sci.* 54(7), 4538–4547 (2013).

64 Thomson JA, Itskovitz-Eldor J, Shapiro SS *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* 282(5391), 1145–1147 (1998).

65 Tsai RJ, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N. Engl. J. Med.* 343(2), 86–93 (2000).

66 Kawashima M, Kawakita T, Satake Y, Higa K, Shimazaki J. Phenotypic study after cultivated limbal epithelial transplantation for limbal stem cell deficiency. *Arch. Ophthalmol.* 125(10), 1337–1344 (2007).

67 Baradaran-Rafii A, Ebrahimi M, Kanavi MR *et al.* Midterm outcomes of autologous cultivated limbal stem cell transplantation with or without penetrating keratoplasty. *Cornea* 29(5), 502–509 (2010).

68 Rama P, Matuska S, Paganoni G, Spinelli A, De Luca M, Pellegrini G. Limbal stem-cell therapy and long-term corneal regeneration. *N. Engl. J. Med.* 363(2), 147–155 (2010).

69 Pauklin M, Fuchsluger TA, Westekemper H, Steuhl KP, Meller D. Midterm results of cultivated autologous and allogeneic limbal epithelial transplantation in limbal stem cell deficiency. *Dev. Ophthalmol.* 45, 57–70 (2010).

70 Sangwan VS, Basu S, Vemuganti GK *et al.* Clinical outcomes of xeno-free autologous cultivated limbal epithelial transplantation: a 10-year study. *Br. J. Ophthalmol.* 95(11), 1525–1529 (2011).

71 Desousa JL, Daya S, Malhotra R. Adnexal surgery in patients undergoing ocular surface stem cell transplantation. *Ophthalmology* 116(2), 235–242 (2009).

72 Utheim TP. Limbal epithelial cell therapy: past, present, and future. *Methods Mol. Biol.* 1014, 3–43 (2013).

73 Ricardo JR, Cristovam PC, Filho PA *et al.* Transplantation of conjunctival epithelial cells cultivated *ex vivo* in patients with total limbal stem cell deficiency. *Cornea* 32(3), 221–228 (2013).

74 Nishida K, Yamato M, Hayashida Y *et al.* Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium. *N. Engl. J. Med.* 351(12), 1187–1196 (2004).

75 Nakamura T, Takeda K, Inatomi T, Sotozono C, Kinoshita S. Long-term results of autologous cultivated oral mucosal epithelial transplantation in the scar phase of severe ocular surface disorders. *Br. J. Ophthalmol.* 95(7), 942–946 (2011).

76 Melles GR. Posterior lamellar keratoplasty: DLEK to DSEK to DMEK. *Cornea* 25(8), 879–881 (2006).

77 Mimura T, Amano S, Usui T *et al.* Transplantation of corneas reconstructed with cultured adult human corneal endothelial cells in nude rats. *Exp. Eye Res.* 79(2), 231–237 (2004).

78 Mimura T, Yamagami S, Yokoo S *et al.* Cultured human corneal endothelial cell transplantation with a collagen sheet in a rabbit model. *Invest. Ophthalmol. Vis. Sci.* 45(9), 2992–2997 (2004).

79 Koizumi N, Sakamoto Y, Okumura N *et al.* Cultivated corneal endothelial cell sheet transplantation in a primate model. *Invest. Ophthalmol. Vis. Sci.* 48(10), 4519–4526 (2007).

80 Ksander BR, Kolovou PE, Wilson BJ *et al.* ABCB5 is a limbal stem cell gene required for corneal development and repair. *Nature* 511, 353–357 (2014).