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Dry Eye and Ocular Surface Disease

Stem cell-based therapy for treating limbal stem cells deficiency A review of different strategies

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Abstract

The self renewal capability of limbal epithelial stem (LEST) cells is fundamental to the maintenance and healing of corneal epithelium. Limbal stem cell deficiency (LSCD), due to dysfunction or loss of LEST cells, therefore presents as persistent epithelial defects, corneal vascularization, conjunctivalization etc. Stem cell-based therapy, in its simplest form – limbal autograft, has been used successfully for more than a decade. For bilateral LSCD, similar approaches with limbal allografts have been unsuccessful largely due to strong immune rejection. Therefore, as an alternate strategy for treating bilateral LSCD, ex vivo expansion of the remaining LEST cells or autologous stem cells sourced from other potential sites is being explored. Different culture systems (with and without xenobiotic supplements) using substrates like amniotic membrane or fibrin gels have been used successfully for ex vivo LEST cell maintenance and reproduction by imitating the stem cell niche. This paper is organized into sections reviewing the LEST cells, LSCD and various stem cell-based approaches for treating LSCD and discussing future direction and challenges.

Keywords: Limbal epithelial stem (LEST), Limbal stem cell deficiency (LSCD), Limbal autograft

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Introduction

The Limbal epithelial stem (LEST) cells have self renewal capabilities and therefore, allow the corneo-scleral limbus to serve as a barrier. LEST cells divide and differentiate into corneal epithelial cells; replacing them completely every 9–12 months. Thus, LEST cells shield the cornea from encroachment of the conjunctival cells and blood vessels, maintaining ocular surface integrity and functionality. Deficiency of LEST cells inhibits ocular surface restoration and may result in ocular irritation, epiphora, blepharospasm, photophobia, pain, severe visual impairment and even corneal blindness.[1]

Stem cell-based therapy, in its preliminary form, was brought into clinical use for corneal limbal stem cells deficiency (LSCD) more than a decade ago. This paper is aimed at reviewing the different strategies, either in use or under development, for the identification of LEST cells, etiology and diagnosis of LSCD and stem cell-based approaches for treating LSCD; analyzing their strengths, limitations, and challenges; and understanding the direction of future research.

Corneal limbal epithelial stem cells

LEST niche

Within the corneo-scleral limbus,[2] the LEST cell niche is thought to be located in the palisades of Vogt (PV). Palisades of Vogt are radially oriented fibrovascular structures located from limbo-corneal junction to 1–2 mm from it that are prominent in the upper and lower quadrants. Their morphology is

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believed to create an optimal microenvironment filled with stem cell nutrients and growth factors that not only supports stem cell growth but also regulates the process of cell division.[3–5] Different growth factors, such as insulin-like growth factor, fibrocyte growth factor, epidermal growth factor etc., have been identified as vital elements of molecular networks responsible for the stem cell modulation.[6,7] *Dickkopf (DKK)* family member, DKK2, mediates repression of the Wnt/β-catenin signaling pathway and is essential to promote differentiation and stratification of the corneal epithelial progenitor cells.[8] Further, Takac's group proposed the role of the molecule CXC chemokine receptor type 4 (CXCR4) and DKK4 in the differentiation and maintenance of corneal epithelial cells by whole human genome expression microarrays.[9] Moreover, potential cross talk between the LEST and the nervous system has been described.[10]

Multiple findings that led to the understanding that LEST cells are located in the PV. Early observations of the centripetal migration of pigment from limbus toward the central cornea led to the understanding that the corneal-scleral limbus was the source of LEST cells.[11] This idea was further supported by follow-up studies in which the basal epithelial cells residing in the PV were found to lack the differentiation related marker K3, but were reactive with the 3H-thymidine and BrdU which are used to mark the slow cycling cells.[12–14] Other evidence supporting the idea comes from the description of the limbal epithelial crypt (LEC) in the PV. LEC are solid cords of epithelial cells appearing to emerge from the posterior end of the limbal palisades that stain with Tenascin C and ATP-binding cassette transporter G2 (ABCG2) transporter protein and extend into the underlying substantia propria, radially parallel to the palisades or circumferentially along the limbus at right angles to the palisades.[15–17]

Identification of LEST cells

A number of studies were undertaken to explore specific LEST cell markers. Several markers were proposed to be expressed in the limbal basal cells including: enzymes, like α-enolase, cytochrome oxidase, carbonic anhydrase; growth factor receptors such as the epidermal growth factor (EGF) receptor and transforming growth factor β (TGF- β) receptor I and II; cell cycle mediators, like cyclins D and E, and ABCG2; the transcription factor ΔNp63.[18–23] However, there is no consensus on a specific marker for the LEST cells. Therefore, expression of putative stem cell markers and lack of differentiation related markers (K3/K12) emerged as an alternative strategy for the identification of LEST cells.[24–31] Other strategies include cell morphology,[24] clone formation assay[32] and DNA retention study.[12,13]

Corneal epithelium regeneration

The LEST cells usually remain in a quiescent state (slow cycling and G_0) in the niche, divide to be the transit amplifying (TA) cells after activation which then move to the superior layer and migrate across the limbus toward the peripheral cornea as young TA cells. Meanwhile, the more mature TA cells with reduced proliferative potential reside in the central corneal area, finally becoming the terminal cells. This scheme of stem cell division/differentiation is described as ''stem cell – transit amplifying (TA) cell – terminal cell''. In response to injury, replicative potential of TA cells increases and the cell cycle length is shortened to increase the replication efficiency.[14,33]

Limbal stem cell deficiency

Limbal stem cell deficiency (LSCD) occurs due to loss or dysfunction of LEST cells, characterized by the failure of epithelium regeneration, and therefore, causing persistent corneal epithelial defects or chronic recurrent corneal erosion, chronic corneal inflammation, corneal vascularization, conjunctivalization, corneal graft rejection and secondary infection.

Etiology

The etiology of LSCD can be classified as– hereditary or primary and acquired or secondary. Briefly, hereditary causes include aniridia, keratitis associated with multiple endocrine deficiency, ectrodactyly-ectodermal dysplasia-clefting syndrome, keratitis-ichthyosis-deafness syndrome and dyskeratosis congenita. Acquired causes include contact lens wear, chemical and thermal burns, inflammatory ocular surface disease (Stevens–Johnson syndrome, toxic epidermal necrolysis, ocular cicatricial pemphigoid, Mooren's ulcer, chronic limbitis, neurotrophic keratopathy, chronic bullous keratopathy, pterygium) and systemic diseases (diabetes, vitamin A deficiency, graft-versus-host disease, rosacea).

Diagnosis

The diagnosis of LSCD is based on the detection of the goblet cells in the corneal epithelium which implies conjunctival epithelial ingrowth due to the diminished barrier function of the LEST cells. Impression cytology (IC) is one of the classic approaches in which Periodic Acid Schiff is used to highlight the goblet cells. In the new PCR-strip-based diagnostic system, the expression of goblet cell specific protein mucin 5AC is detected at mRNA level using PCR-reverse dot blot.[34] Recently, an application of the confocal microscope was reportedly used to detect LSCD. Compared with IC, confocal microscopy is considered to be a safer and faster but more expensive method.[35,36]

Stem cell based therapy

Stem cell-based therapy has been performed for over a decade for the LSCD with outstanding outcomes. In its simplest form, conjunctival limbal autograft (CLAU) has been successfully used in the treatment of unilateral LSCD. However, there is a concern of inducing LSCD in the donor eye; therefore leading to its modification involving a smaller source tissue in conjunction with in vivo expansion. While these methods have been successful for patients with unilateral LSCD, similar approaches with conjunctival limbal allograft (due to bilateral LSCD) have been largely unsuccessful due to a high frequency of immune rejection. Therefore, ex vivo expansion of remaining LEST cells or stem cells sourced from other potential sites is being explored as a more viable solution for bilateral LSCD. In this part we review different stem cell based therapies to treat LSCD.

Corneal limbal epithelial stem cell transplantation

Autograft transplantation

Conjunctival limbal autograft (CLAU). In CLAU, conjunctival limbal graft from the fellow donor eye is transplanted to the affected recipient eye. Since the first report in 1989 by Kenyon and Tseng, CLAU has become a widely accepted technique in the management of unilateral total LSCD.[37] Various studies reported overwhelmingly successful improvements of vision and corneal surface integrity.[38–46] In the original description of the procedure, two strips were removed from the contralateral eye, each consisting of 120° limbus and about 5 mm adjacent to the conjunctiva. This led to a significant concern about the risk of inducing LSCD in the donor eye. How small a size is sufficient to successfully initiate the corneal surface rebuilding is still unknown. To decrease the risk, the application of smaller size grafts was reported in other studies. While Ahmad Kheirkhah reported that one 60° CLAU successfully restored the entire corneal surface in chemical burn-induced LSCD,[41] other studies seemed to indicate that smaller size grafts were associated with a corresponding decrease in the success rate.[44,45] Being an autograft, an obvious advantage of CLAU is that there is no risk of immune rejection and therefore, no need for any immunosuppression. However, the risk of inducing LSCD in the donor eye, particularly in a patient whose fellow eye also has subclinical LSCD, limits applications of CLAU.[47]

Simple limbal epithelial transplantation (SLET). A newer strategy combining CLAU and in vivo expansion utilizing amniotic membrane transplantation seems to improve the success rate of smaller grafts.[42,48] Amniotic membrane seems to inhibit inflammation and provide a supportive niche for the transplanted LEST cells. In 2012, Sanqwan VS[51] described a novel surgical technique termed SLET. The study included 6 patients with chemical burn induced LSCD and follow-up duration ranging between 7.5 and 12 months. In this procedure, a small 2×2 mm strip was removed from the fellow eye and chopped into pieces. Then the tiny pieces were seeded on the amniotic membrane (AM) covered cornea. Complete reconstruction with epithelialized, avascular and stable corneal surface was observed after 6 weeks in all 6 recipient eyes. Improved vision outcomes were recorded in 4 of the 6 recipient eyes (66.6%). The 6 patients involved in the study showed physical signs of LSCD including 360° absence of the PV, dull and irregular corneal epithelium, superficial corneal vascularization, persistent epithelial defects or conjunctival overgrowth on the corneal surface; however, the pathological evidence provided for the LSCD confirmation was limited. Subsequently, successful applications of SLET in previously failed pediatric limbal transplantation for ocular surface burns and a lime injury-induced LSCD case were reported by Bhalekar[49] and Vazirani[50] in 2013. While short term outcomes seem promising, long-term success rates are yet to be determined.[51]

Corneal stem cell allograft transplantation

Allograft transplantation is for the patients suffering from bilateral total LSCD or whose fellow eye is not suitable as a graft source. Generally, allograft transplantation includes cadaveric Keratolimbal allograft (KLAL) and living-related conjunctivallimbal allograft (Lr-CLAL).[52–54] Due to high risk of immune rejection, both the methods offer poor long-term outcomes when compared with the autograft transplantation. Severe bilateral LSCD is often accompanied by immune system diseases like SJS, toxic epidermal necrolysis, or severe trauma injury, which are typically associated with immune system hyperactivity resulting in a higher risk of immune reaction. Therefore, high frequency of immune rejection of the KLAL is not only due to extensive vascularization and Langerhans' cell-enriched areas contained in the grafts but also to the overactive immune system of the recipient. Several reports have demonstrated that even under continuous immunosuppressive drug therapy such as Cyclosporin A, steroid, FK506, mycophenolate mofetil, the failure rate of KLAL increases dramatically beyond 2–3 years of follow-up.

Cultured limbal epithelial transplantation (CLET)

LEST cell culture strategies. Due to the high risk of rejection associated with allografts, ex vivo expansion of remaining LEST cells is a theoretically preferable solution for bilateral LSCD. Based on the elements contained, culture systems used for the ex vivo expansion can be divided into either a ''xenobiotic culture system'' or a ''xenobiotic-free culture system''.

Briefly, the xenobiotic elements include murine derived-3T3 feeder cells, fetal calf serum and various animal-derived growth factors. A number of studies have shown these elements to be beneficial for ex vivo LEST cell maintenance and reproduction by imitating the stem cell niche; however, safety concerns of potential transmission of contagious agents, tumorigenesis, or immune rejection has redirected the focus of research on xenobiotic-free culture systems. Human dermal fibroblasts have been found to be a promising replacement for 3T3 feeder layer in LEST cells culture system.[55,56] Meanwhile, Aboulghassem Shahdadfar and Meeta Pathak demonstrated the successful use of autologous human serum to support the expansion of LEST cells in vitro[57] and improve clinical outcomes.[57,58] Notably, autologous human serum was the only supplement added into the culture medium in these two studies. Human cord blood serum has also been successfully used as limbal stem cell culture supplement.[59] In addition, successful establishment of a tissue-engineered corneal epithelium is highly dependent on the underlying scaffold. Several materials such as human amniotic membrane,[60] fibrin gels,[61–63] collagen, [64–66] keratin films,[67–69] silk fibroin films,[70] chitosan hydrogels,[71] siloxane-hydrogel contact lens,[72] Polystyrene,[68] and nanofiber scaffold[73] have been tested as scaffolds. All of these materials have been found to support the growth of LEST cells in vivo, but only human amniotic membrane and fibrin gels have been investigated in clinical studies with positive outcomes.

Clinical outcomes. A review of the literature reveals that several studies evaluated the clinical outcomes of CLET in LSCD. Table 1 summarizes the outcomes of the studies in the past 13 years (average follow up duration of $\geqslant$12 months). While there are several studies documenting short-term effectiveness, studies with longer follow up duration, that can reveal long term prognosis after transplantation, are limited in number.[62,74–82] The data shown in Table 1 provides several insights: (1) either amniotic membrane or Fibrin has been approved to be a qualified carrier in CLET, associated with a

Table 1. Literature summary of CLET for LSCD.

Authors	Year	Case	FU (months)	Substrate	3T3	Biopsy size	Success rate (%)
I R Schwab[81]	2000	14	6–19	AM	Y	2 mm^2	71.40
Rama P[62]	2001	18	27	Fibrin	Y	1–2 mm^2	77.80
Tsai RI[79]	2003	6	15 ± 2	AM	N	1 × 2 mm^2	77.80
Takahiro[75]	2004	1	19	AM	Y	3 mm^2	100
Sangwan[78]	2006	86	18.3	AM	N	1 × 2 mm^2	73.10
Gisold[74]	2010	6	24	Fibrin	Y	1–2 mm^2	82.30
Rama P[77]	2010	112	up to 10 years	Fibrin	Y	1–2 mm^2	76.70
Giorgio[82]	2012	16	12–50	Fibrin	Y	1–2 mm^2	62.6[△],18.7[#]
Pathak[*76]	2012	9	11–28	AM	N	1.5 × 2.5 × 0.25 mm^3	55.60
Zakaria N[*80]	2014	18	24	AM	N	1 × 2 mm^2	67

CLET = cultured limbal epithelial transplantation; FU = follow up; N = no; Y = yes;
[*] Animal free culture system (human serum is the only supplement in the culture medium).
[△] Complete success restoration.
[#] Partial restoration.

high success rate; (2) 3T3 is widely used to support the limbal stem cell growth in vitro however xenobiotic free culture systems without 3T3 feeder layer also obtained fairly high success rate in CLET; (3) Even though xenobiotic free culture systems had a lower rate of success in comparison with CLET with the xenobiotic culture systems, it is still a promising option. Furthermore, Rama P and co-workers revealed a positive correlation between the percentage of p63 positive cells in the grafts and the success rate of CLET. Cultures containing more than 3% p63 positive stem cells were associated with successful transplantation in 78% patients.[77] This novel notion provides a criterion to measure the quality of stem cells cultured in the grafts which may be useful to increase the success rate of CLET in future.

Other alternative stem cell sources

In cases of extensive bilateral LSCD, where it may not be possible to source any limbal epithelium stem cells, alternative stem cell sources may be helpful.

Mesenchymal stem cells (MSCs)

MSCs, originally isolated from tissues such as bone marrow, adipose,[83] heart, spleen,[84,85] cord blood,[86] oral,[85] are well known for their multipotency and plasticity. Numerous studies have shown that MSCs have the capability to promote wound healing; the eye is no different from other tissues in this respect. Recently, human corneal limbal stroma-derived MSCs with the potential for epithelial trans-differentiation have been detected in several studies.[87–91] Besides, successful reconstruction of damaged cornea using bone marrow derived MSCs, adipose derived MSCs, and umbilical MSCs by subconjunctival injection, graft transplantation and intravenous infusion has been observed in animal studies and lab works. The mechanisms involved were considered to be associated with the immunomodulatory function and differentiation.[92–101] To the best of our knowledge, few clinical or pre-clinical studies have been reported until now.

Dental pulp immature stem cells (hIDPSCs)

hIDPSCs express various markers including those specific for MSCs, embryonic stem cells and neural cells. In 2009, Monteiro and co-workers demonstrated that hIDPSC share markers similar to corneal limbal epithelial stem cells, such as ABCG2 and P63.[102] One year later, a report regarding the restoration of corneal epithelium in the mild chemical burn rabbit model of LSCD using hIDPSCs grafts was published and suggested the therapeutic potential of hIDPSC in the treatment of LSCD in future.[103]

Embryonic stem cells (ESCs)

ESCs have been widely accepted as a promising cell source in the tissue engineering based treatment for tissue regeneration. In vitro, the ESCs in different species such as humans, rabbits and mice were found to have the capability to differentiate into corneal epithelial-like cells in the microenvironment, mimicking the corneal epithelial stem cell niche.[104–107] Recently, Hanson demonstrated that the human ESCs can be transferred to partially wounded human cornea in vitro, and grown on the Bowman's membrane to form corneal epithelial-like cells.[108] Furthermore, rapid construction of embryonic stem cell sheets with or without scaffold has been described in 2013, which provided greater possibility for transplantation in future.[109,110]

Hair follicle bulge cells

Hair follicle bulge is an essential niche for keratinocyte stem cells (KSCs).[111,112] Blazejewska and co-workers reported successful trans-differentiation of hair follicle stem cells into corneal epithelial-like cells in 2009.[113] After 2 years, the same group used the transgenic mice as animal model to evaluate the therapeutic potential of hair follicle bulge in vitro. Isolated autologous hair follicle bulge cells were expanded on a fibrin carrier in vitro, and then transferred into the mice with LSCD. The immunostaining results suggested that the hair follicle bulge cells contribute to the reconstruction of corneal epithelium by crossing the lineage boundaries and terminally differentiating into corneal epithelial-like cells.[114]

Oral mucosal epithelium

In contrast to the alternative stem cells described above, the safety and efficiency of oral mucosal epithelium based transplantation has been evaluated clinically. Several groups from Japan demonstrated that cultured oral mucosal epithelium can be used to reconstruct the corneal epithelium in animal models as well as patients with LSCD due to chemical injury and SJS. Nakamura's study[115] included 19 eyes of 17 patients. Follow up duration ranged from 36 to 90 months. Visual improvement was detected in 95% eyes at 6 months after surgery. After 30 months, the visual improvement percentage gradually abated to 53%. Another study with fewer numbers of patients (7 eyes) reported that just one

eye developed partial LSCD at the 47th month after transplantation.[116] One year later, Burillon's study involving 26 eyes of 25 patients reported a success rate of 64% at 12 months after surgery.[117]

Conclusion

LSCD is a well known disease that affects epithelium maintenance due to the dysfunction or loss of limbal stem cells. Stem cell-based treatments are targeted at replacing the abnormal limbal stem cells with homologous and/or heterologous stem cells. Compared to other surgical procedures, more clinical data with CLAU and KLAL have been collected and analyzed over the past decades. The success rate of CLAU is higher than KLAL as the autografts, for obvious reasons, are better than the allografts for transplantation. The development of tissue engineering techniques led to the emergence of CLET as a promising therapeutic approach in clinical ophthalmology. However, limitations in the isolation of autologous limbal stem cells from the patients suffering from serious bilateral LSCD necessitated the exploration of alternative autologous stem cell sources. Currently, to enhance the stem cell expansion and transplantation efficiency, research is being focused on optimizing the culture conditions; exploring novel scaffolds supporting stem cell proliferation, maintenance and differentiation; and evaluating the therapeutic potential of different kinds of autologous stem cells.

However, several different barriers still remain. The characteristics and anatomical structure of the limbal stem cell niche are still obscure and the specific markers for limbal stem cells remain uncertain. Besides, the molecular networks responsible for modulation of the stem cell bio-behaviors are unclear. More work needs to be done to address these important concerns and make stem cell-based therapy for treating limbal stem cell deficiency more successful.

Conflict of interest

The authors declared that there is no conflict of interest.

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References

1. Dua HS, Azuara-Blanco A. Limbal stem cells of the corneal epithelium. *Surv Ophthalmol* 2000;**44**:415–25.
2. Van Buskirk EM. The anatomy of the limbus. *Eye (Lond)* 1989;**3**(Pt 2):101–8.
3. Goldberg MF, Bron AJ. Limbal palisades of Vogt. *Trans Am Ophthalmol Soc* 1982;**80**:155–71.
4. Lagali N, Eden U, Utheim TP, et al. In vivo morphology of the limbal palisades of Vogt correlates with progressive stem cell deficiency in aniridia-related keratopathy. *Invest Ophthalmol Vis Sci* 2013;**54**:5333–42.
5. Townsend WM. The limbal palisades of Vogt. *Trans Am Ophthalmol Soc* 1991;**89**:721–56.
6. Trosan P, Svobodova E, Chudickova M, et al. The key role of insulin-like growth factor I in limbal stem cell differentiation and the corneal wound-healing process. *Stem Cells Dev* 2012;**21**:3341–50.
7. Klenkler B, Sheardown H. Growth factors in the anterior segment: role in tissue maintenance, wound healing and ocular pathology. *Exp Eye Res* 2004;**79**:677–88.
8. Mukhopadhyay M, Gorivodsky M, Shtrom S, et al. Dkk2 plays an essential role in the corneal fate of the ocular surface epithelium. *Development* 2006;**133**:2149–54.
9. Takacs L, Toth E, Losonczy G, et al. Differentially expressed genes associated with human limbal epithelial phenotypes: new molecules that potentially facilitate selection of stem cell-enriched populations. *Invest Ophthalmol Vis Sci* 2011;**52**:1252–60.
10. Bath C, Muttuvelu D, Emmersen J, et al. Transcriptional dissection of human limbal niche compartments by massive parallel sequencing. *PLoS One* 2013;**8**:e64244.
11. Davanger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature* 1971;**229**:560–1.
12. Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 1986;**103**:49–62.
13. Cotsarelis G, Cheng SZ, Dong G, et al. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell* 1989;**57**:201–9.
14. Lehrer MS, Sun TT, Lavker RM. Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. *J Cell Sci* 1998;**111**(Pt 19):2867–75.
15. Dua HS, Shanmuganathan VA, Powell-Richards AO, et al. Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. *Br J Ophthalmol* 2005;**89**:529–32.
16. Shanmuganathan VA, Foster T, Kulkarni BB, et al. Morphological characteristics of the limbal epithelial crypt. *Br J Ophthalmol* 2007;**91**:514–9.
17. Yeung AM, Schlotzer-Schrehardt U, Kulkarni B, et al. Limbal epithelial crypt: a model for corneal epithelial maintenance and novel limbal regional variations. *Arch Ophthalmol* 2008;**126**:665–9.
18. Hayashi K, Kenyon KR. Increased cytochrome oxidase activity in alkali-burned corneas. *Curr Eye Res* 1988;**7**:131–8.
19. Pellegrini G, Dellambra E, Golisano O, et al. P63 identifies keratinocyte stem cells. *Proc Natl Acad Sci USA* 2001;**98**:3156–61.
20. Steuhl KP, Thiel HJ. Histochemical and morphological study of the regenerating corneal epithelium after limbus-to-limbus denudation. *Graefes Arch Clin Exp Ophthalmol* 1987;**225**:53–8.
21. Zieske JD, Bukusoglu G, Yankauckas MA, et al. Alpha-enolase is restricted to basal cells of stratified squamous epithelium. *Dev Biol* 1992;**151**:18–26.
22. Zieske JD, Hutcheon AE, Guo X, et al. TGF-beta receptor types I and II are differentially expressed during corneal epithelial wound repair. *Invest Ophthalmol Vis Sci* 2001;**42**:1465–71.
23. Zieske JD, Takahashi H, Hutcheon AE, Dalbone AC. Activation of epidermal growth factor receptor during corneal epithelial migration. *Invest Ophthalmol Vis Sci* 2000;**41**:1346–55.
24. Arpitha P, Prajna NV, Srinivasan M, Muthukkaruppan V. High expression of p63 combined with a large N/C ratio defines a subset of human limbal epithelial cells: implications on epithelial stem cells. *Invest Ophthalmol Vis Sci* 2005;**46**:3631–6.
25. Budak MT, Alpdogan OS, Zhou M, et al. Ocular surface epithelia contain ABCG2-dependent side population cells exhibiting features associated with stem cells. *J Cell Sci* 2005;**118**:1715–24.
26. de Paiva CS, Chen Z, Corrales RM, et al. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells* 2005;**23**:63–73.
27. Di Iorio E, Barbaro V, Ruzza A, et al. Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci USA* 2005;**102**:9523–8.
28. Kiritoshi A, SundarRaj N, Thoft RA. Differentiation in cultured limbal epithelium as defined by keratin expression. *Invest Ophthalmol Vis Sci* 1991;**32**:3073–7.
29. Schlotzer-Schrehardt U, Kruse FE. Identification and characterization of limbal stem cells. *Exp Eye Res* 2005;**81**:247–64.
30. Watanabe K, Nishida K, Yamato M, et al. Human limbal epithelium contains side population cells expressing the ATP-binding cassette transporter ABCG2. *FEBS Lett* 2004;**565**:6–10.
31. Wolosin JM, Budak MT, Akinci MA. Ocular surface epithelial and stem cell development. *Int J Dev Biol* 2004;**48**:981–91.
32. Pellegrini G, Golisano O, Paterna P, et al. Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. *J Cell Biol* 1999;**145**:769–82.
33. Thoft RA, Friend J. The X, Y, Z hypothesis of corneal epithelial maintenance. *Invest Ophthalmol Vis Sci* 1983;**24**:1442–3.

34. Garcia I, Etxebarria J, Merayo-Lloves J, et al. Novel molecular diagnostic system of limbal stem cell deficiency based on MUC5AC transcript detection in corneal epithelium by PCR-reverse dot blot. *Invest Ophthalmol Vis Sci* 2013;**54**:5643–52.
35. Goldberg MF. In vivo confocal microscopy and diagnosis of limbal stem cell deficiency. Photographing the palisades of Vogt and limbal stem cells. *Am J Ophthalmol* 2013;**156**:205–6.
36. Nubile M, Lanzini M, Miri A, et al. In vivo confocal microscopy in diagnosis of limbal stem cell deficiency. *Am J Ophthalmol* 2013;**155**:220–32.
37. Kenyon KR, Tseng SC. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology* 1989;**96**:709–22 discussion 22-3.
38. Dogru M, Tsubota K. Survival analysis of conjunctival limbal grafts and amniotic membrane transplantation in eyes with total limbal stem cell deficiency. *Am J Ophthalmol* 2005;**140**:305–6.
39. Dua HS, Azuara-Blanco A. Autologous limbal transplantation in patients with unilateral corneal stem cell deficiency. *Br J Ophthalmol* 2000;**84**:273–8.
40. Ivekovic R, Tedeschi-Reiner E, Novak-Laus K, et al. Limbal graft and/or amniotic membrane transplantation in the treatment of ocular burns. *Ophthalmologica* 2005;**219**:297–302.
41. Kheirkhah A, Raju VK, Tseng SC. Minimal conjunctival limbal autograft for total limbal stem cell deficiency. *Cornea* 2008;**27**:730–3.
42. Meallet MA, Espana EM, Grueterich M, et al. Amniotic membrane transplantation with conjunctival limbal autograft for total limbal stem cell deficiency. *Ophthalmology* 2003;**110**:1585–92.
43. Mittal V, Sangwan VS, Fernandes M, Thomas R. Survival analysis of conjunctival limbal grafts and amniotic membrane transplantation in eyes with total limbal stem cell deficiency. *Am J Ophthalmol* 2006;**141**:599–600 author reply.
44. Moldovan SM, Borderie V, Baudrimont M, Laroche L. Treatment of unilateral limbal stem cell deficiency syndrome by limbal autograft. *J Fr Ophtalmol* 1999;**22**:302–9.
45. Rao SK, Rajagopal R, Sitalakshmi G, Padmanabhan P. Limbal autografting: comparison of results in the acute and chronic phases of ocular surface burns. *Cornea* 1999;**18**:164–71.
46. Santos MS, Gomes JA, Hofling-Lima AL, et al. Survival analysis of conjunctival limbal grafts and amniotic membrane transplantation in eyes with total limbal stem cell deficiency. *Am J Ophthalmol* 2005;**140**:223–30.
47. Tseng SCG, EMEaMADP. Stem cell trasplantation for ocular surface diseases. In: Tasman W, Jaeger Edward A, editors. Duane's clinical ophthalmology on CD-ROM, vol. 2. Baltimore, MD: Lippincott Williams & Wilkins; 2006 [chapter 34].
48. Pires RT, Chokshi A, Tseng SC. Amniotic membrane transplantation or conjunctival limbal autograft for limbal stem cell deficiency induced by 5-fluorouracil in glaucoma surgeries. *Cornea* 2000;**19**:284–7.
49. Bhalekar S, Basu S, Lal I, Sangwan VS. Successful autologous simple limbal epithelial transplantation (SLET) in previously failed paediatric limbal transplantation for ocular surface burns. BMJ Case Rep 2013;2013.
50. Vazirani J, Basu S, Sangwan V. Successful simple limbal epithelial transplantation (SLET) in lime injury-induced limbal stem cell deficiency with ocular surface granuloma. BMJ Case Rep 2013;2013.
51. Sangwan VS, Basu S, MacNeil S, Balasubramanian D. Simple limbal epithelial transplantation (SLET): a novel surgical technique for the treatment of unilateral limbal stem cell deficiency. *Br J Ophthalmol* 2012;**96**:931–4.
52. Huang T, Wang Y, Zhang H, et al. Limbal allografting from living-related donors to treat partial limbal deficiency secondary to ocular chemical burns. *Arch Ophthalmol* 2011;**129**:1267–73.
53. Lam DS, Young AL, Leung AT, et al. Limbal stem cell allografting from related live donors for corneal surface reconstruction. *Ophthalmology* 2000;**107**:411–2.
54. Rao SK, Rajagopal R, Sitalakshmi G, Padmanabhan P. Limbal allografting from related live donors for corneal surface reconstruction. *Ophthalmology* 1999;**106**:822–8.
55. Lu R, Bian F, Lin J, et al. Identification of human fibroblast cell lines as a feeder layer for human corneal epithelial regeneration. *PLoS One* 2012;**7**:e38825.
56. Sharma SM, Fuchsluger T, Ahmad S, et al. Comparative analysis of human-derived feeder layers with 3T3 fibroblasts for the ex vivo expansion of human limbal and oral epithelium. *Stem Cell Rev* 2012;**8**:696–705.
57. Shahdadfar A, Haug K, Pathak M, et al. Ex vivo expanded autologous limbal epithelial cells on amniotic membrane using a culture medium with human serum as single supplement. *Exp Eye Res* 2012;**97**:1–9.
58. Albert R, Vereb Z, Csomos K, et al. Cultivation and characterization of cornea limbal epithelial stem cells on lens capsule in animal material-free medium. *PLoS One* 2012;**7**:e47187.
59. Chakraborty A, Dutta J, Das S, Datta H. Effect of cord blood serum on ex vivo human limbal epithelial cell culture. *J Ocul Biol Dis Inform* 2012;**5**:77–82.
60. Shimazaki J, Aiba M, Goto E, et al. Transplantation of human limbal epithelium cultivated on amniotic membrane for the treatment of severe ocular surface disorders. *Ophthalmology* 2002;**109**:1285–90.
61. Han B, Schwab IR, Madsen TK, Isseroff RR. A fibrin-based bioengineered ocular surface with human corneal epithelial stem cells. *Cornea* 2002;**21**:505–10.
62. Rama P, Bonini S, Lambiase A, et al. Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. *Transplantation* 2001;**72**:1478–85.
63. Talbot M, Carrier P, Giasson CJ, et al. Autologous transplantation of rabbit limbal epithelia cultured on fibrin gels for ocular surface reconstruction. *Mol Vis* 2006;**12**:65–75.
64. Dravida S, Gaddipati S, Griffith M, et al. A biomimetic scaffold for culturing limbal stem cells: a promising alternative for clinical transplantation. *J Tissue Eng Regen Med* 2008;**2**:263–71.
65. McIntosh Ambrose W, Salahuddin A, So S, et al. Collagen Vitrigel membranes for the in vitro reconstruction of separate corneal epithelial, stromal, and endothelial cell layers. *J Biomed Mater Res B Appl Biomater* 2009;**90**:818–31.
66. Takezawa T, Ozaki K, Nitani A, et al. Collagen vitrigel: a novel scaffold that can facilitate a three-dimensional culture for reconstructing organoids. *Cell Transplant* 2004;**13**:463–73.
67. Borrelli M, Reichl S, Feng Y, et al. In vitro characterization and ex vivo surgical evaluation of human hair keratin films in ocular surface reconstruction after sterilization processing. *J Mater Sci Mater Med* 2013;**24**:221–30.
68. Feng Y, Borrelli M, Meyer-Ter-Vehn T, et al. Epithelial wound healing on keratin film, amniotic membrane and polystyrene in vitro. *Curr Eye Res* 2014;**39**:561–70.
69. Reichl S, Borrelli M, Geerling G. Keratin films for ocular surface reconstruction. *Biomaterials* 2011;**32**:3375–86.
70. Bray LJ, George KA, Ainscough SL, et al. Human corneal epithelial equivalents constructed on *Bombyx mori* silk fibroin membranes. *Biomaterials* 2011;**32**:5086–91.
71. Grolik M, Szczubialka K, Wowra B, et al. Hydrogel membranes based on genipin-cross-linked chitosan blends for corneal epithelium tissue engineering. *J Mater Sci Mater Med* 2012;**23**:1991–2000.
72. Di Girolamo N, Chui J, Wakefield D, Coroneo MT. Cultured human ocular surface epithelium on therapeutic contact lenses. *Br J Ophthalmol* 2007;**91**:459–64.
73. Zajicova A, Pokorna K, Lencova A, et al. Treatment of ocular surface injuries by limbal and mesenchymal stem cells growing on nanofiber scaffolds. *Cell Transplant* 2010;**19**:1281–90.
74. Colabelli Gisoldi RA, Pocobelli A, Villani CM, et al. Evaluation of molecular markers in corneal regeneration by means of autologous cultures of limbal cells and keratoplasty. *Cornea* 2010;**29**:715–22.
75. Nakamura T, Inatomi T, Sotozono C, et al. Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease. *Acta Ophthalmol Scand* 2004;**82**:468–71.
76. Pathak M, Cholidis S, Haug K, et al. Clinical transplantation of ex vivo expanded autologous limbal epithelial cells using a culture medium with human serum as single supplement: a retrospective case series. *Acta Ophthalmol* 2013;**91**:769–75.
77. Rama P, Matuska S, Paganoni G, et al. Limbal stem-cell therapy and long-term corneal regeneration. *N Engl J Med* 2010;**363**:147–55.
78. Sangwan VS, Matalia HP, Vemuganti GK, et al. Clinical outcome of autologous cultivated limbal epithelium transplantation. *Indian J Ophthalmol* 2006;**54**:29–34.
79. Tsai RJ, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med* 2000;**343**:86–93.
80. Zakaria N, Possemiers T, Dhubhghaill SN, et al. Results of a phase I/II clinical trial: standardized, non-xenogenic, cultivated limbal stem cell transplantation. *J Transl Med* 2014;**12**:58.

81. Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease(1). *Am J Ophthalmol* 2000;**130**:543–4.
82. Marchini G, Pedrotti E, Pedrotti M, et al. Long-term effectiveness of autologous cultured limbal stem cell grafts in patients with limbal stem cell deficiency due to chemical burns. *Clin Exp Ophthalmol* 2012;**40**:255–67.
83. Zannettino AC, Paton S, Arthur A, et al. Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. *J Cell Physiol* 2008;**214**:413–21.
84. Hoogduijn MJ, Crop MJ, Peeters AM, et al. Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. *Stem Cells Dev* 2007;**16**:597–604.
85. Zhang Q, Shi S, Liu Y, et al. Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis. *J Immunol* 2009;**183**:7787–98.
86. Oh W, Kim DS, Yang YS, Lee JK. Immunological properties of umbilical cord blood-derived mesenchymal stromal cells. *Cell Immunol* 2008;**251**:116–23.
87. Branch MJ, Hashmani K, Dhillon P, et al. Mesenchymal stem cells in the human corneal limbal stroma. *Invest Ophthalmol Vis Sci* 2012;**53**:5109–16.
88. Hashmani K, Branch MJ, Sidney LE, et al. Characterization of corneal stromal stem cells with the potential for epithelial transdifferentiation. *Stem Cell Res Ther* 2013;**4**:75.
89. Holan V, Javorkova E, Trosan P. The growth and delivery of mesenchymal and limbal stem cells using copolymer polyamide 6/12 nanofiber scaffolds. *Methods Mol Biol* 2013;**1014**:187–99.
90. Li GG, Zhu YT, Xie HT, et al. Mesenchymal stem cells derived from human limbal niche cells. *Invest Ophthalmol Vis Sci* 2012;**53**:5686–97.
91. Lim MN, Hussin NH, Othman A, et al. Ex vivo expanded SSEA-4+ human limbal stromal cells are multipotent and do not express other embryonic stem cell markers. *Mol Vis* 2012;**18**:1289–300.
92. Du Y, Roh DS, Funderburgh ML, et al. Adipose-derived stem cells differentiate to keratocytes in vitro. *Mol Vis* 2010;**16**:2680–9.
93. Jia Z, Jiao C, Zhao S, et al. Immunomodulatory effects of mesenchymal stem cells in a rat corneal allograft rejection model. *Exp Eye Res* 2012;**102**:44–9.
94. Joyce NC, Harris DL, Markov V, et al. Potential of human umbilical cord blood mesenchymal stem cells to heal damaged corneal endothelium. *Mol Vis* 2012;**18**:547–64.
95. Liu H, Zhang J, Liu CY, et al. Bone marrow mesenchymal stem cells can differentiate and assume corneal keratocyte phenotype. *J Cell Mol Med* 2012;**16**:1114–24.
96. Nieto-Miguel T, Galindo S, Reinoso R, et al. In vitro simulation of corneal epithelium microenvironment induces a corneal epithelial-like cell phenotype from human adipose tissue mesenchymal stem cells. *Curr Eye Res* 2013;**38**:933–44.
97. Oh JY, Lee RH, Yu JM, et al. Intravenous mesenchymal stem cells prevented rejection of allogeneic corneal transplants by aborting the early inflammatory response. *Mol Ther* 2012;**20**:2143–52.
98. Park SH, Kim KW, Chun YS, Kim JC. Human mesenchymal stem cells differentiate into keratocyte-like cells in keratocyte-conditioned medium. *Exp Eye Res* 2012;**101**:16–26.
99. Reza HM, Ng BY, Gimeno FL, et al. Umbilical cord lining stem cells as a novel and promising source for ocular surface regeneration. *Stem Cell Rev* 2011;**7**:935–47.
100. Rohaina CM, Then KY, Ng AM, et al. Reconstruction of limbal stem cell deficient corneal surface with induced human bone marrow mesenchymal stem cells on amniotic membrane. *Transl Res* 2014;**163**:200–10.
101. Yao L, Li ZR, Su WR, et al. Role of mesenchymal stem cells on cornea wound healing induced by acute alkali burn. *PLoS One* 2012;**7**:e30842.
102. Monteiro BG, Serafim RC, Melo GB, et al. Human immature dental pulp stem cells share key characteristic features with limbal stem cells. *Cell Prolif* 2009;**42**:587–94.
103. 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* 2010;**51**:1408–14.
104. 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* 2007;**25**:1145–55.
105. Chan AA, Hertsenberg AJ, Funderburgh ML, et al. Differentiation of human embryonic stem cells into cells with corneal keratocyte phenotype. *PLoS One* 2013;**8**:e56831.
106. 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* 2004;**45**:4320–6.
107. Kumagai Y, Kurokawa MS, Ueno H, et al. Induction of corneal epithelium-like cells from cynomolgus monkey embryonic stem cells and their experimental transplantation to damaged cornea. *Cornea* 2010;**29**:432–8.
108. Hanson C, Hardarson T, Ellerstrom C, et al. Transplantation of human embryonic stem cells onto a partially wounded human cornea in vitro. *Acta Ophthalmol* 2013;**91**:127–30.
109. Zhang W, Yang W, Liu X, et al. Rapidly constructed scaffold-free embryonic stem cell sheets for ocular surface reconstruction. *Scanning* 2013.
110. Zhu J, Zhang K, Sun Y, et al. Reconstruction of functional ocular surface by acellular porcine cornea matrix scaffold and limbal stem cells derived from human embryonic stem cells. *Tissue Eng Part A* 2013;**19**:2412–25.
111. Ohyama M. Hair follicle bulge: a fascinating reservoir of epithelial stem cells. *J Dermatol Sci* 2007;**46**:81–9.
112. Ohyama M, Terunuma A, Tock CL, et al. Characterization and isolation of stem cell-enriched human hair follicle bulge cells. *J Clin Invest* 2006;**116**:249–60.
113. 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* 2009;**27**:642–52.
114. 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* 2011;**29**:57–66.
115. Nakamura T, Takeda K, Inatomi T, et al. Long-term results of autologous cultivated oral mucosal epithelial transplantation in the scar phase of severe ocular surface disorders. *Br J Ophthalmol* 2011;**95**:942–6.
116. Liu J, Sheha H, Fu Y, et al. Oral mucosal graft with amniotic membrane transplantation for total limbal stem cell deficiency. *Am J Ophthalmol* 2011;**152**(739–47):e1.
117. Burillon C, Huot L, Justin V, et al. Cultured autologous oral mucosal epithelial cell sheet (CAOMECS) transplantation for the treatment of corneal limbal epithelial stem cell deficiency. *Invest Ophthalmol Vis Sci* 2012;**53**:1325–31.