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REVIEW

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Targeting limbal epithelial stem cells: master conductors of corneal epithelial regeneration from the bench to multilevel theranostics

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Abstract

The cornea is the outermost layer of the eye and plays an essential role in our visual system. Limbal epithelial stem cells (LESCs), which are localized to a highly regulated limbal niche, are the master conductors of corneal epithelial regeneration. Damage to LESCs and their niche may result in limbal stem cell deficiency (LSCD), a disease confused ophthalmologists so many years and can lead to corneal conjunctivalization, neovascularization, and even blindness. How to restore the LESCs function is the hot topic for ocular scientists and clinicians around the world. This review introduced LESCs and the niche microenvironment, outlined various techniques for isolating and culturing LESCs used in LSCD research, presented common diseases that cause LSCD, and provided a comprehensive overview of both the diagnosis and multiple treatments for LSCD from basic research to clinical therapies, especially the emerging cell therapies based on various stem cell sources. In addition, we also innovatively concluded the latest strategies in recent years, including exogenous drugs, tissue engineering, nanotechnology, exosome and gene therapy, as well as the ongoing clinical trials for treating LSCD in recent five years. Finally, we highlighted challenges from bench to bedside in LSCD and discussed cutting-edge areas in LSCD therapeutic research. We hope that this review could pave the way for future research and translation on treating LSCD, a crucial step in the field of ocular health.

Keywords Limbal epithelial stem cells, Niche microenvironment, Limbal stem cell deficiency, Stem cell regeneration, Cell-based therapy, Clinical translation

Introduction

The cornea is the outermost clear and avascular tissue of the eye surface, which plays a key role in maintaining transparency and visual function by allowing light to reach the ocular epithelium and activate nerve impulses in the retina [1]. It is reported that corneal disease is the third leading cause of blindness, and more than 10 million people are suffering from blindness worldwide [2]. As a protective barrier of the eye surface, the corneal epithelium is continuously renewed by limbal epithelial stem cells (LESCs) [3]. LESCs are adult stem cells with a slow cell cycle, low differentiation rate, asymmetric division, high proliferation potential, and strong self-renewal

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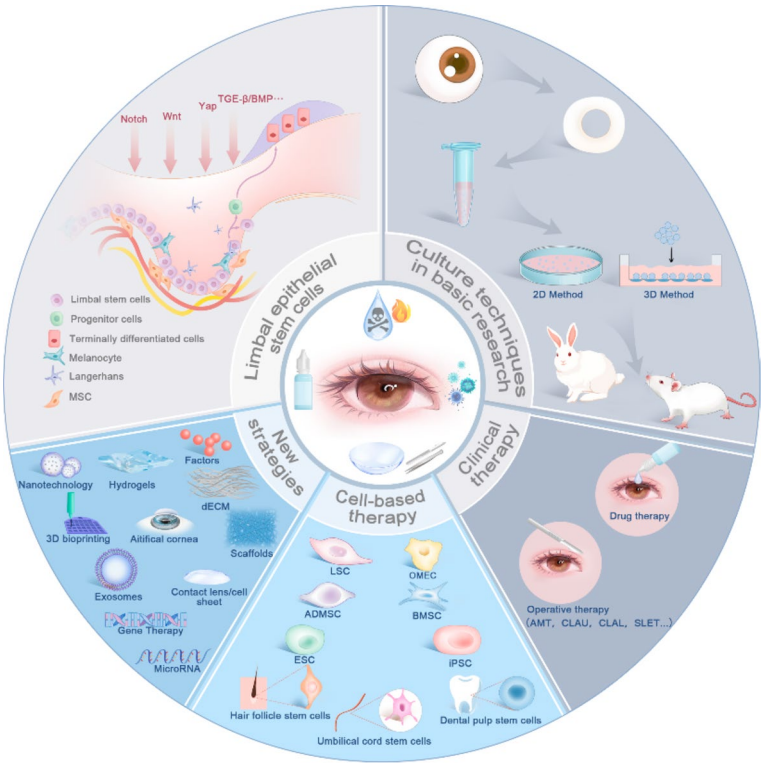
Graphical Abstract



ability [4, 5]. Anatomically, LESCs are located in crypt-like structures (such as limbal epithelial crypts and Vogt palisades) in human limbal tissues, known as the niche [6]. The niche is a highly controlled marginal microenvironment with unique physical, autocrine, paracrine, and multicellular properties specifically designed to shelter and regulate LESCs [7].

Dysfunction or loss of LESCs and their niche can lead to limbal stem cell deficiency (LSCD), a disease characterized by invasion of the conjunctival epithelium into the cornea and failure of epithelial wound healing [8]. Corneal opacity, pain, visual loss, and blindness are consequences of LSCD [9]. Successful treatment of LSCD depends on accurate diagnosis and staging of the disease and requires restoration of functional LESCs and their niche. At present, there are various clinical treatment options for LSCD, including amniotic membrane transplantation (AMT), conjunctival limbal autograft (CLAU), conjunctival limbal allograft (CLAL), simple limbal epithelial transplantation (SLET) etc. [10] Stem cell therapies from multiple sources, exogenous factors, decellularized matrix, tissue engineering, nanotechnology, exosomes (Exos), gene therapy and microRNA (miRNA) have brought new ideas for the treatment of LSCD as well.

In this review, we first introduced the anatomy, microenvironment and regulatory pathways of LESCs. These are the basis for designing comprehensive and innovative therapeutic strategies for ocular surface reconstruction. Furthermore, various techniques for isolating and culturing LESCs used in LSCD therapeutic research were summarized, which could elucidate additional therapeutic potentials for the future. Subsequently, we described in detail the diagnosis and treatments of LSCD, focusing on the emerging cell therapies based on various stem cell sources. We also innovatively summarized the latest research progress regarding LESCs in recent years, such as exogenous drugs, tissue engineering, nanotechnology, exosome, gene therapy and miRNA, and their related clinical translations. Finally, we concluded the current challenges in the field and proposed future development directions to provide inspiration for basic and clinical research on LSCD and other ocular diseases.

Anatomical location and characteristics of limbal epithelial stem cells

The corneal epithelium is a self-renewing tissue that is maintained and continuously renewed by stem cells uniquely located in the basal epithelial layer of the limbus, namely the 1.5–2.5 mm transition zone between the cornea and sclera, which was proposed by Schermer et al.

in 1986 [11, 12]. Since then, the biological characteristics of LESCs have attracted considerable attention.

LESCs are relatively undifferentiated cells with small morphology (diameter ≤ 12 μm), high nuclear cytoplasmic ratio, slow cell cycle, and higher self-renewal, proliferation, and colony formation abilities [4, 5]. Under steady-state conditions, LESCs maintain a slow cell cycle and increase their proliferative capacity after injury. More importantly, corneal epithelial stem cells are thought to be involved in the renewal and regeneration of the corneal epithelium [13]. LESCs divide asymmetrically, producing a transient amplifying cell and a stem cell, and therefore, there is no net change to the stem cell population. These transient amplifying cells can either migrate centrally along the basal epithelium or move to the surface to gradually become terminally differentiated cells of the stratified corneal epithelium. This process has been summarized as the XYZ hypothesis (Fig. 1), which explained how LESCs maintain a balance between corneal epithelial cell number and homeostasis [14].

Identification and biomarkers of limbal epithelial stem cells

Like other somatic stem cells, LESCs highly express stem cell markers, including transporters (such as ABCG2 and ABCB5), cytokeratin (such as CK5, CK14, CK15, CK17, and CK19), transcription factors (such as ΔNp63α, Pax6, Bmi-1, and C/EBPδ), cell adhesion molecules such as CD44, and receptors (such as N-cadherin, integrin α9 and β1, and frizzed 7) [4, 15–18]. The loss of the differentiation markers CK3, CK12, connexin 43, involucrin, and stage-specific embryonic antigen 4 (SSEA4) also provides new ideas for the identification of LESCs [15, 19]. In addition, single-cell analysis has proposed new markers such as SOX17, TSPAN7, and GPHA2 [20, 21].

Currently, the transcription factor p63 is considered an important marker expressed in the nuclei of LESCs located in the basal layer of the limbal epithelium [22]. There are six isoforms of p63, three of which are thought to be full-length (Tap63) and the other three lacking the N-terminal domain (ΔNp63). ΔNp63α is the most abundant isoform in the limbus and has the highest correlation with LESCs [22, 23]. The percentage of $p63\alpha^{positive}$ cells is a significant indicator to evaluate the culture system of LESCs. Rama et al. showed that LESC grafts containing more than 3% $p63\alpha^{positive}$ cells had a 76% success rate of transplantation [24]. However, the nuclear localisation of p63 weakens its use in the isolation of surface marker-based LESCs.

The membrane-expressing protein ABCG2 is another characteristic of LESCs, and its expression in a small subset of limbal basal epithelial cells has been previously reported [25]. In 2014, Ksander et al. [16] demonstrated that ABCB5 also marked a slowly cycling population of basal cells that expressed ΔNp63α at the corneal margin. The rabbit model of LSCD treated by $ABCB5^{+}$ cell transplantation can restore a clear avascular cornea without LSCD characteristics over time, while $ABCB5^{-}$ cell transplantation cannot [26]. At present, researchers are using cell surface markers such as ABCB5 to purify limbal epithelial stem cells to improve their efficacy [27].

CK5, CK14, CK15, CK17, and CK19 are associated with undifferentiated epithelial cells of the human limbal epithelium and are therefore considered as markers of undifferentiated corneal epithelial cells [15, 20]. It should be noted that CK19, although present in the basal limbal epithelium, can also be found in the conjunctiva and cornea and is thus not a good marker for undifferentiated cells [28]. Another marker of LESCs is CD44, a

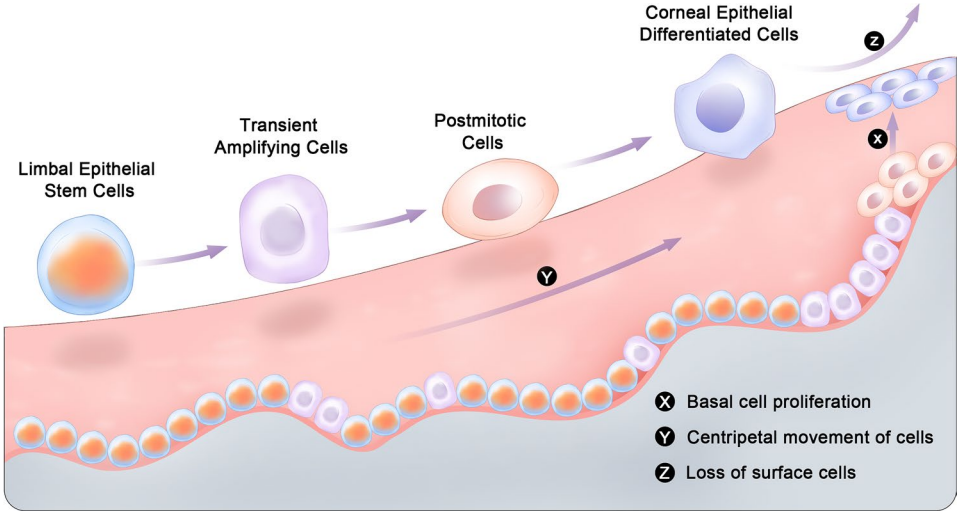


Fig. 1 The "XYZ" hypothesis. X: proliferation of basal epithelial cells; Y: the centripetal movement of peripheral cells; Z: epithelial cell loss from the surface

homing-related cell adhesion molecule that is a powerful tool for the molecular characterisation of LESCs [18].

Basal cells of the limbal epithelium are relatively undifferentiated and lack the expression of differentiation markers, such as CK3, CK12, involucrin, and connexin 43 [25]. Since CK3 is also expressed in the conjunctival epithelium, CK12 is a more specific corneal epithelial marker than CK3 [29]. SSEA4 is commonly used as a cell surface marker to identify pluripotent human embryonic stem cells (hESCs) [30]. Notably, SSEA4 is highly expressed in differentiated human corneal epithelial cells, and $SSEA4^-$ limbal epithelial cells contain a high proportion of LESCs. Therefore, SSEA4 can be used as a negative marker for LESCs [19].

Recently, an innovative study [31] showed that extracellular miR-6723-5p can be used as a biomarker for the human LESC population, where the level of miR-6723-5p in medium is correlated with the number of $\Delta P63\alpha^{bright}$ stem cells. However, due to the relatively small sample size and the possibility that other miRNAs may have specific regulatory patterns, further studies are warranted.

Although the expression patterns of these indices are generally consistent with the presence of stem cells, a clear phenotype of ocular epithelial stem cells corresponding to true stem cell activity has not yet been established. The specific reasons for this are as follows: (1) They are preferentially, but not exclusively, expressed in LESCs. (2) Early differentiated cells still possess stem cell markers and exhibit intermediate features between stem cells and differentiated cells until stem cell markers are downregulated in response to the expression of the differentiated phenotype. (3) The enzymatic digestion process of isolating LESCs and some immunostaining steps may result in alterations in the surface antigens of some proteins, such as ABCB5 and ABCG2 [4]. Therefore, using a single marker to define LESCs is not feasible, and a set of markers and phenotypic descriptions are required to define cell populations with stem cell characteristics.

The niche of limbal epithelial stem cells

LESCs primarily exist in the niche provided by crypt-like structures in the human limbus (including limbal epithelial crypts and Vogt's palisades), which is not only a protective environment in response to external signals but also a necessary factor for maintaining the stem cell characteristics of LESCs [6].

The main components of the limbal niche include extracellular matrix (ECM), chemical molecules, and limbal niche cells such as melanocytes, mesenchymal cells (MSCs), nerve cells (especially Schwann cells), immune cells, and vascular cells (Fig. 2) [32]. Their interaction maintains the metabolic homeostasis of LESCs by releasing a large number of growth factors and soluble molecules and provides dynamic support for the proliferation, migration, stemness maintenance, and differentiation of LESCs [13, 33].

Melanocytes

Limbal melanocytes (LMels) are neural crest-derived cells that are mainly distributed in the limbal basal layer, and their main function is to transfer melanosomes containing melanin to LESCs to protect them from ultraviolet radiation and free radical damage [34, 35]. The degree of pigmentation correlates with the differentiation status

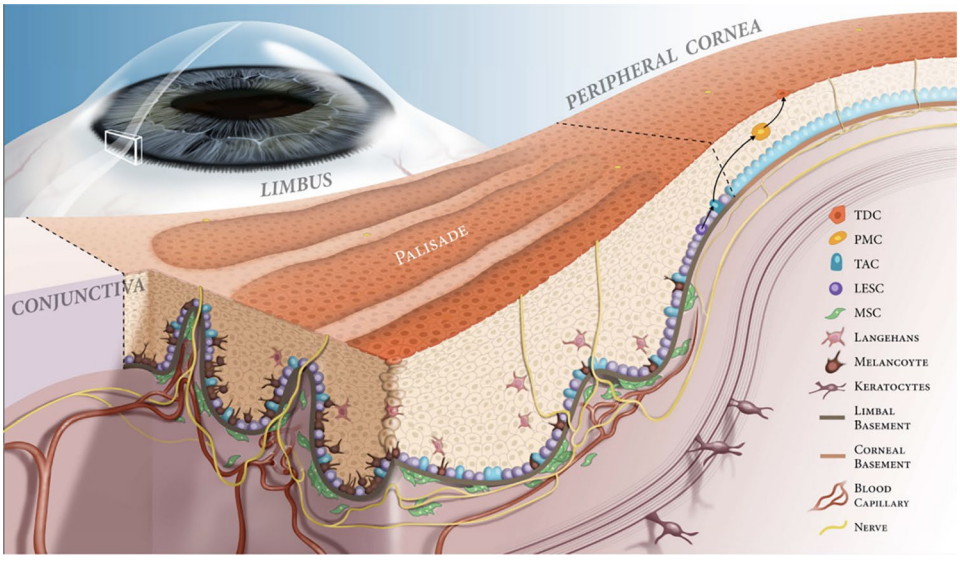


Fig. 2 The limbal niche of LESCs. The corneoscleral limbus comprises limbal epithelial crypts and the Palisades of Vogt. LESCs are in close contact with niche cells including melanocytes, Langerhans cells, nerve cells and mesenchymal stem cells (MSCs). Abbreviations: TDC, terminally differentiated cell, PMC, post-mitotic cell, TAC, transient amplifying cell, LESC, limbal epithelial stem cell, MSC, mesenchymal stem cell. Reproduced with permission. Copyright 2019, Ocular Surface

of the LESCs, among which the most pigmented population tend to be the most immature progenitor cells [36]. It is reported that there is an interaction between melanocytes and $CK19^{positive}$ cells, and that CD44 is highly enriched in melanocytes as a receptor [37, 38]. Importantly, LMels also play multiple non-normative functions in the homeostasis of niche by maintaining the LESC phenotype, supporting LESCs expansion in vitro, regulating immune responses, and controlling angiogenesis.

At present, laminin-332, laminin-511, and laminin-511-E8 have been reported to promote the adhesion, migration, differentiation, and proliferation of epidermal melanocytes [39]. Polisetti et al. [40] used CD90 and CD117 as selective markers to isolate and enrich melanocytes from human corneal margins. These findings provide guidance for further research on the regulatory mechanisms of melanocytes and their unique roles in the limbus.

Mesenchymal stem cells

Limbal mesenchymal stem cells (LMSCs), also known as limbal niche cells, interact with LESCs via various chemical and signal transduction pathways. These include chondroitin sulfate (6C3 motif), IL-6/STAT3, SDF-1/CXCR4, BMP/Wnt, vimentin and aquaporin-1 [41]. Further, LMSCs are attractive tools for clinical applications because of their potent immunomodulatory, antiinflammatory, and anti-angiogenic properties [42].

Intercellular contacts and paracrine growth factor secretion are other communication mechanisms involved in the interaction between LESCs and LMSCs [43]. Studies have shown that LMSCs co-cultured with LESCs in vitro could maintain LESCs in a progenitor-like state by secreting elevated levels of IL6 [44]. Stromal cells located directly below the margin may had a greater capacity to support LESCs growth than cells located in deeper margins of the stroma [45].

Moreover, mesenchymal cells from different sources can support LESC growth. These MSCs may produce factors that favour the maintenance of the LESCs phenotype [46]. However, the efficiency at which each MSC supports LESC expansion varies. The selection of MSCs as feeder cells for clinical applications depends on their availability and their ability to support the growth of stem cell populations.

Schwann cells

Schwann cells (SCs) are the main glial cells in the peripheral nervous system and are divided into myelinated and non-myelinated SCs per their axons [47]. Like all peripheral nerve fibres, corneal axons interact with SCs, which support axonal maintenance and conduction and play a key role in tissue regeneration [48].

Comparative analysis of the mouse limbus using scRNA-seq suggested that corneal SCs were potential sources of multiple trophic factors, including nerve growth factors. There were multiple paracrine interactions between SCs and LESCs, as well as between SCs and MSCs. These findings suggested a novel role for limbal SCs in mediating nerve-dependent corneal epithelial renewal [49]. In the future, the potential role of SCs in regulating the limbal niche function needs to be further investigated.

Immune cells

Immune cells, such as B cells, NK/T cells, mononuclear macrophages, dendritic cells, mast cells, and Langerhans cells, are the niche cells of LESCs and play important roles in maintaining the undifferentiated LESC phenotype and corneal homeostasis [11, 21].

For example, by suppressing T cells with topical application of the corticosteroid dexamethasone, LESCs showed a significant reduction in Cd63 and Gpha2 expression and an increase in cell proliferation. Moreover, corneal epithelial wound healing was delayed in mice lacking T cells. These results suggested that T cells, as niche cells, play key roles in maintaining LESCs quiescence, controlling epithelial thickness and wound healing [11, 50]. Dou et al. [38] depicted six human limbal tissues by using single-cell RNA sequencing and found that monocyte macrophages had the most interaction links with LESCs, such as Jagged1-Notch2, IL24/DLL1-Notch2, and DLL1-Notch3 pairs, suggesting that monocyte macrophages were involved in the regulation of LESCs through the Notch signaling pathway. In addition, several ligand receptors related to the Notch and Wnt signaling pathways existed between dendritic cells and LESCs, which played important roles in regulating corneal epithelial wound repair [51]. Langerhans cells are characterised by the expression of MSR1, VSIG4, and PTPRC, which should be explored in future studies [52].

Vascular endothelial cells

Vascular endothelial cells are important niche cells of LESCs. It has been reported that vascular endothelial cells are highly associated with the classical Wnt signaling pathway and may be involved in the regulation of the limbal niche. Vascular endothelial cells express characteristic markers, such as COL4A2, PECAM1, CD31, and CLDN5, which may represent human limbal vasculature [52]. In the future, the potential role of vascular endothelial cells in regulating the limbal niche function needs to be further investigated.

Extracellular matrix

The ECM is a key component of the LESC niche and is mainly composed of water, polysaccharides, hyaluronic

acid, collagen, and proteins [53]. In addition, the ECM is not only a supporting scaffold but also mediates intercellular communication, and drives cell fate decisions [54]. Biological and biomechanical interactions between stem cells and their ECM have been proven to influence cell fate and phenotype through a variety of regulatory pathways including YAP/TAZ, β-catenin signaling and ΔNp63 pathway [55]. Moreover, ECM also provides a new direction for the development of decellularized matrices.

In summary, the detailed interpretation of the components of niche will help researchers and clinicians to better understand limbal biology and design therapeutic plans for treating LSCD.

Signal pathways involved in the regulation of limbal epithelial stem cells

LESCs are regulated by various signaling pathways. Notch, Wnt, YAP, transforming growth factor-β (TGF-β)/BMP, and Sonic hedgehog (SHH) signaling pathways play significant roles in the regulation of different types of LESCs [56]. Understanding the regulatory pathways may provide a deeper perspective for basic research and the development of new therapeutic strategies.

Notch signaling pathway

Notch signaling is a developmentally conserved signaling pathway that participates in a variety of biological processes and controls many processes, such as cell proliferation, differentiation, apoptosis, corneal epithelial homeostasis, and wound healing [57, 58]. The Notch receptor family (Notch 1–4) is a heterodimeric transmembrane protein that interacts with delta-like ligand 1 (DLL1), delta-like ligand 3 (DLL3), delta-like ligand 4 (DLL4), Jaggded-1, and Jaggded-2, resulting in the cleavage of the cytoplasmic domain of the receptor and release of the Notch intracellular domain (NICD) [59]. NICD translocates to the nucleus and forms transcription complexes with other proteins, thereby regulating the transcription of genes, such as HES1, HES5, and HEY1, which are present in the basal layer of the human limbal epithelium and are involved in cell-cell interactions between LESCs and their niche cells [57]. The Notch signaling pathway is illustrated in detail in Fig. 3.

Notably, the Notch signaling pathway is a characteristic signaling pathway of LESCs and is significantly related to the proliferation and differentiation abilities of LESCs [60]. One of the signaling pathways involved in LESC stemness is the Jagged 1/Notch pathway. A previous study [60] showed that activation of Notch by Jagged 1

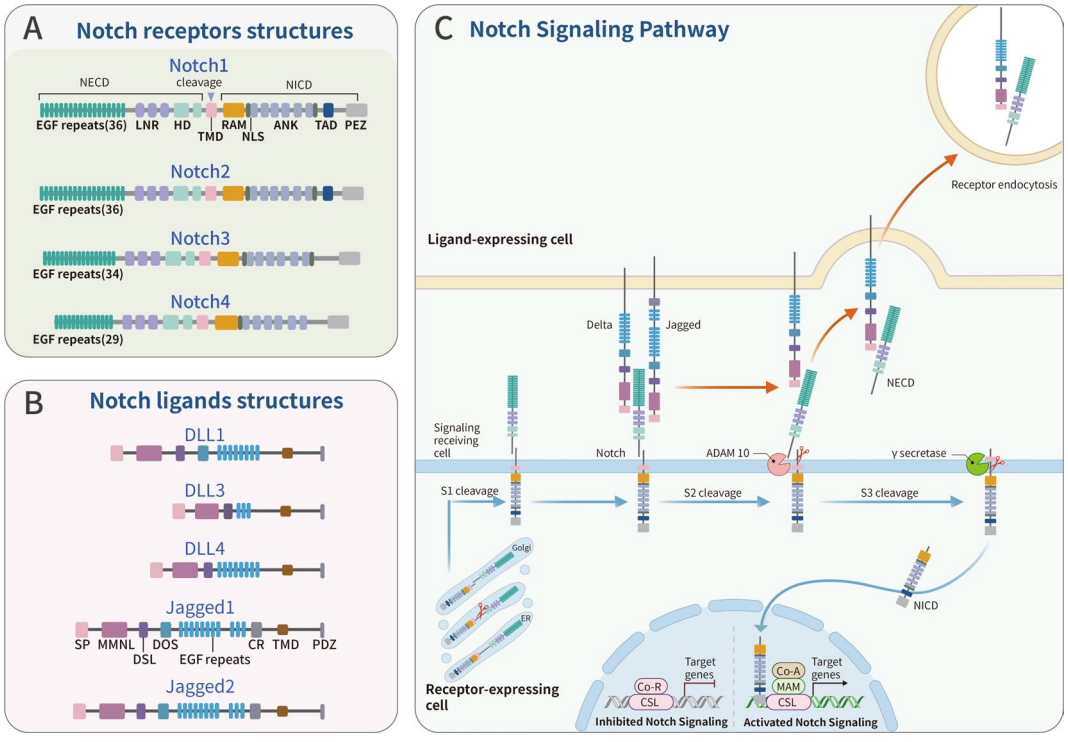


Fig. 3 (**A**) The structures of four Notch receptors (Notch 1, Notch 2, Notch 3, Notch 4) (**B**) The structures of five Notch ligands (DLL1, DLL3, DLL4, Jagged 1, Jagged 2) (**C**) Notch receptors are generated in the ER and trafficked to the Golgi apparatus, and then transported to the cell membrane to form heterodimers; Notch ligands from signal-sending cells bind to the NECD of signal-receiving cells. The binding triggers cleavage by ADAM and then γ-secretase, which releases activated NICD; Activated NICD enters the nucleus and bind with CSL and recruit MAMLs, releasing corepressors, recruiting coactivators, and thus promoting the transcription of Notch target genes (e.g., Hes1, Hey1)

resulted in increased proliferation and decreased differentiation of human corneal epithelial cells. Whereas, by using immobilized Jagged 1 to mediate Notch activation, Gonzalez et al. [61] detected decreased number of small and p63α^{bright} cells, as well as the increased proportion of CK12^{+} cells, indicating the enhanced differentiation ability of human limbal epithelial cells. The differences between the two studies may be attributed to the different methods and levels of activation of Jagged 1 and the Notch signaling pathway, which should be further investigated.

In addition, Notch signaling proteins, including the Notch 1 receptor and HES1/HEY1 target genes, are present in the basal layer of the human limbal epithelium and involved in cell-cell interactions between LESCs and their niche cells [62]. During the repairment of mice corneal epithelial cells, Notch1 signaling maintained cell fate by regulating the expression of retinol-binding protein-1 and vitamin A metabolism [63]. Conditioned inactivation of Notch 1 in adult mice induced hyperplasia and keratosis of the corneal epithelium by activating the β-catenin pathway, thereby mimicking epidermal differentiation [64]. Nevertheless, it is unknown whether the limbal niche is altered when epithelial expression of Notch 1 is perturbed.

DAPT and SAHM1 are Notch signaling inhibitors in LESCs [65, 66]. In rats, inhibition of Notch signaling also reduced the number of LESCs, increased the stemness marker p63α and decreased the differentiation marker CK12 expression of LESCs [67]. Similar results have been obtained in human LESCs [61].

In conclusion, the core molecular components of the Notch signaling pathway appear to have specific expression patterns in the limbus, which may potentially mark the LESC population in this region. At present, little is known about the regulation of Notch signaling in LESCs, and there is much contradictory information, which still require further investigation.

Wnt signaling pathway

Wnt signaling includes a complex set of signal transduction pathways that play key roles in the proliferation, differentiation, and apoptosis of LESCs, as well as stemness and homeostasis maintenance [68]. Wnt signaling pathway is divided into two major categories: the canonical Wnt pathway (β-catenin dependent) and the non-canonical Wnt pathway (β-catenin independent). The non-canonical Wnt pathway also includes the planar cell polarity (PCP) pathway and Ca2^{+} pathways [69, 70] (Fig. 4).

Among these, 19 Wnt ligands, 10 Frizzled (Fzd) receptors, 4 Dickkopf (DKK) inhibitors, and 5 inhibitor-secreted Fzd-related proteins have been reported in humans [71]. Notably, Wnt2, Wnt6, Wnt11, and Wnt16b are preferentially expressed in the limbus where LESCs are located and may play unique roles [72]. For example, Zhao et al. [73] demonstrated that Wnt16b promoted the proliferation and self-renewal of limbal epithelial cells through the CXCR4/MEK/ERK signaling pathway in a mouse corneal wound healing model. In addition, inhibitors of canonical Wnt signaling, such as DKK1, Fzd-related protein B, secreted frizzled-related protein 5,

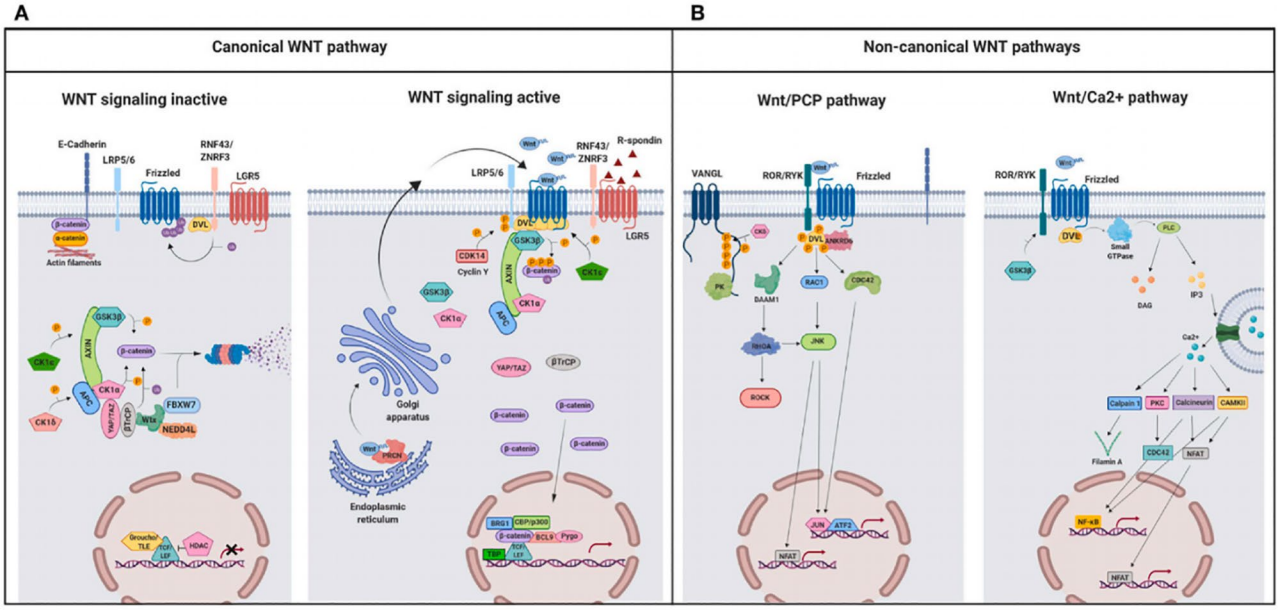


Fig. 4 Schematic diagram of the Wnt signaling pathways. The Wnt signaling pathways can be divided into the canonical Wnt pathway and the non-canonical Wnt pathways (including Wnt /PCP pathway and Wnt/Ca2^{+} pathway). Reproduced with permission. Copyright 2023, Progress in Retinal and Eye Research

and Wnt inhibitory factor-1, are also upregulated in the limbus compared to the cornea [72, 74]. Moreover, Fzd receptors, as key components of Wnt signaling, are preferentially expressed in the limbus as well, and the knockdown of Fzd7 receptor resulted in decreased stemness marker expression in human LESCs [75]. Wnt ligands bind to various receptors and coreceptors to initiate downstream signaling.

Canonical Wnt signaling involves the binding of Wnt ligands to a receptor complex formed by Fzd and LRP5/6. Receptor recruitment leads to the accumulation of cytoplasmic β-catenin. After transport into the nucleus, β-catenin binds to T cell factor/lymphocyte enhancer factor transcription factor to initiate transcription of Wnt/β-catenin target genes such as c-Myc, c-Jun, cyclin D1, and regulate many cellular processes [76, 77]. Nakatsu et al. [72] reported that activation of Wnt/β-catenin signaling increased the proliferation and colony-forming efficiency of primary human limbal epithelial stem cells (hLESCs), with high expression levels of the stemness marker Np63α and low expression levels of differentiation marker CK12. In contrast, Bisevac et al. [78] activated Wnt/β-catenin signaling by treating in vitro expanded hLESCs cultures with GSK-3 inhibitor LY2090314. The results showed increased differentiation of hLESCs, and loss of stemness and proliferation. These conflicting results require further investigation.

The non-canonical Wnt pathway is generally considered independent of β-catenin. Wnt ligands (such as Wnt-4, Wnt-5a, Wnt-5b, and Wnt-11) activate the non-canonical Wnt pathway. It is involved in regulating cell polarity, promoting cell motility and invasion, maintaining stemness, and inhibiting the canonical Wnt/β-catenin signaling pathway [79]. The Wnt/$Ca2^{+}$ pathway activates calmodulin-dependent kinase II (CaMKII) and induces calcium release from the endoplasmic reticulum [80]. Wnt/PCP may use different Fzd coreceptors with ROR, RYK, MuSK, or PTK7 but act on different downstream effectors in different cell types [81]. However, its role in LESC maintenance has only been explored in a few studies and thus remains poorly understood.

Collectively, Wnt signaling is tightly regulated in the LESC niche and a good balance between canonical and non-canonical pathways play an essential role in controlling the biological characteristics of LESCs.

YAP signaling pathway

The Hippo-YAP pathway is a highly conserved signaling pathway that maintains cellular homeostasis under normal conditions and regulates tissue regeneration after injury [82]. The canonical Hippo kinase cascade is initiated by MST1/2, which then phosphorylates and activates LATS1/2. Activated LATS1/2 inactivate YAP and TAZ by inhibiting their nuclear translocation via phosphorylation. When the upstream signal is weak, YAP/TAZ enters the nucleus and interacts with transcription factors to drive or repress target genes [83].

Yap1, a key transcriptional coactivator of the Hippo-YAP signaling pathway, is specifically expressed in LESCs and is essential for maintaining their high proliferative potential [84]. Agrin promotes the proliferation of mouse LESCs by reducing Yap1 phosphorylation and activating the Hippo-YAP signaling pathway [85]. In addition, by constructing corneal epithelial wounds of different sizes in rats, Li et al. found that YAP activation promoted LESCs activation and expansion after large wounds and local epithelial cell reprogramming after small wounds. It also accelerated wound healing of different sizes in the corneal epithelium by regulating cell junctions and the assembly of the cortical F-actin cytoskeleton [86].

It has long been recognized that YAP is a central mediator of a putative mechano-transduction pathway and that YAP/TAZ signaling is regulated by the stiffness of the external ECM [87]. In an in vitro study on the regulation of LESC stemness and cell behaviour based on substrate stiffness, the treatment of bovine LESC with stiffer substrates resulted in a significant loss of stem biomarkers, whereas YAP, a key factor in mechanical transduction, showed increased expression. Therefore, LESCs culture on modified soft substrates is helpful for the treatment of LSCD [55, 88]. Furthermore, the biophysical properties of marginal niches maintain their stemness via YAP. Bhattacharya et al. [89] reported that the unique biomechanical properties of the rim support nuclear localisation and function of YAP-associated proteins. Perturbations in tissue stiffness or YAP activity can affect human LESC function as well as tissue integrity at homeostasis and significantly inhibit stem cell population regeneration. Taken together, biomechanical signaling provides a critical cell fate-determining signal for LESCs during homeostasis and regeneration.

At the same time, the Hippo-YAP signaling pathway also has extensive crosstalk with other signaling pathways, such as Wnt, Notch, TGF/BMP, and inflammatory signaling, making the Hippo pathway a critical sensor of tissue integrity and a direct response to injury [55, 86]. Further studies are needed to determine the exact role of crosstalk between the YAP pathway and other pathways in LESC function.

TGF-β/BMP signaling pathway

The TGF-β signaling pathway is one of the most important signaling pathways regulating the behaviour of ocular tissue cells. Hu et al. [90] showed that mouse corneal epithelial stem cells achieved efficient long-term expansion and maintained stemness under diet-free and serum-free conditions in vitro by inhibiting TGF-β receptor-I-mediated signaling. Kawakita et al. [91] reported

that Smad-mediated TGF-β signaling leads to differentiation and senescence of mouse corneal/limbal epithelial progenitor cells. Han et al. [92] regulated the clonal growth of human LESCs by integrating BMP/Wnt signaling between LESCs and limbal niche cells, which would help elucidate how limbal niche cells regulate LESC stationarity, self-renewal, and fate decisions. However, there are still few studies on TGF-β/BMP in LESCs, and further studies are needed.

Other signaling pathways

Except for the Notch, Wnt, YAP, and TGF-B/BMP signaling pathways, the SHH, p38 MAPK, and integrin-mediated signaling pathways have been shown to regulate the function and phenotype of LESCs. Among them, SHH-mediated signaling activation promotes LESC proliferation and limbal wound healing in mice through gli1 and gli3 mediated cyclin D1 expression [93, 94]. SPARC promoted rabbit LESC proliferation and inhibited the spontaneous differentiation of LESCs through the JNK and p38-MAPK signaling pathways [95]. Integrin signals could activate typical Wnt/ β-catenin signals for transduction [96].

In addition, autophagy is also essential for stem cell homeostasis in various tissues [97]. Autophagic activity was significantly higher in the basal layer of the limbal epithelium than that in the corneal epithelium. When autophagy was blocked, the holographic colony formation ability of limbal epithelial cells was significantly weakened [98, 99]. Furthermore, paracrine factors and their receptors, cell-cell contacts, cell-matrix contacts, and mechanical transduction are also important for LESC self-renewal and fate determination. At present, these related mechanisms are rarely reported and need to be further elucidated.

Taken together, LESC regulation may involve interactions between signaling pathways. Therefore, further studies on the mechanism of LESC regulation are required to maintain a balance between LESC proliferation, differentiation, and quiescence.

Isolation and culture techniques of limbal epithelial stem cells

Understanding LESC biology and marginal niche function will open new avenues for the treatment of LSCD. Obtaining sufficient stem cells is the basis of stem cell therapy, so we outlined various techniques for isolating and culturing LESCs used in LSCD therapeutic research.

Although fresh tissue has been shown to provide better cell yield, viability, and quality, corneoscleral tissue from cadaveric or living donors is commonly used for hLESCs culture [100]. The minimum requirement of corneoscleral tissue to support in vitro expansion of hLESCs is 0.5 mm^2 of cadaveric tissue and 0.3 mm^2 of living tissue [101]. The intact limbal epithelium was stripped of excess sclera, conjunctiva, iris, corneal endothelium, and central cornea to obtain the corneoscleral rings. After thorough washing in DMEM medium containing antibiotics, the limbal epithelium was exfoliated by Dispase II digestion (37 ° C for two hours or 4 ° C one night). After brief treatment of the isolated limbal epithelium with trypsin and EDTA, single-cell suspensions were prepared for seeding [102]. This modality has been used by many researchers as the primary source for subsequent manipulations, including fluorescence-activated cell sorting and culture.

In classical single-cell culture systems, plastic petri dishes and human amniotic membrane (AM) are the typical growth substrates used in standard hLESCs cultures [103]. Mouse 3T3 fibroblasts generally serve as feeder cells that promote the growth and expansion of hLESCs; however, the underlying mechanisms remain unclear. DMEM/F12 supplemented with foetal bovine serum and antibiotics is the most commonly used medium for culturing hLESCs in vitro. In previous studies, many reagents were added to the culture medium to support the growth and expansion of LESCs or to maintain better cell morphology, including epidermal growth factor, adenine, bovine pituitary extract, and transferrin [104, 105].

With the continuous improvement in the understanding of cell-cell and cell-molecular interactions, three-dimensional (3D) artificial niches and culture techniques have been established to simulate the real microenvironment of the limbal recess to better promote the growth and proliferation of hLESCs. “Sandwich culture” is a 3D culture technique established using a transwell co-culture system equipped with a porous membrane at the bottom of the insert to avoid direct cell-cell contact between hLESCs and feeder cells [106]. Real architecture for 3D tissue is another emerging 3D culture technology [107]. In addition, new culture technologies, such as exogenous or feed-free culture systems, the development of new scaffolds, and the synthesis of biocompatible culture substrates, have opened up new avenues for the culture of LESCs [102, 108].

However, insufficient tissue sources, cell contamination, and high costs remain the main challenges in cell culture. Additionally, the mechanisms of cell-cell and cell-molecular interactions need to be further investigated to elucidate the potential of 3D artificial niches and 3D organoids, which is a promising direction for future research.

Limbal stem cell deficiency and its diagnosis, clinical treatment

Dysfunction or loss of LESCs and limbal niche pathology may lead to LESC disorders, and thus, LSCD. According to an analysis of globally reported cases, many factors can cause LSCD, including severe corneal chemical

injury, thermal injury, multiple surgeries, contact lens wear, long-term use of benzalkonium chloride-preserved eye drops, inappropriate medication, and other diseases, such as Stevens-Johnson syndrome, vernal keratoconjunctivitis, aniridia, and graft-versus-host disease [9, 10].

LSCD is a severe disease characterized by corneal neovascularization, opacity, conjunctivalization, chronic inflammation, scarring, and visual loss [8, 9]. Patients with acute LSCD often present with redness and swelling, foreign-body sensation, photophobia, tearing, and vision loss. Biomicroscopy demonstrated conjunctival hyperaemia, irregular corneal epithelium, and alteration or loss of palisades in the Vogt area. In addition, scar formation and extensive neovascularization are also observed on the ocular surface during the chronic phase [109]. Most cases of LSCD are unilateral or bilateral partial, and residual LESCs can be found in eyes with clinical features of LSCD.

Impression cytology is a simple, non-invasive technique that detects goblet cells and conjunctival epithelial cells on the corneal surface and is considered the "gold standard" for the diagnosis of LSCD, but it lacks sensitivity for early lesions or those with mild conjunctivalisation [8]. A positive blot cytologic result can confirm the diagnosis, whereas a negative result cannot rule out the disease. Furthermore, the development of ocular imaging techniques, such as in vivo laser scanning confocal microscopy and anterior segment optical coherence tomography, as well as the detection of molecular markers, has led to a further understanding of the structure of the limbus and improved the accurate diagnosis of LSCD [10, 110, 111].

Clinical management of LSCD varies depending on the severity and extent of involvement. Treatments include symptom control and aetiology in patients with mild and moderate LSCD. For example, small doses of topical steroids, autologous serum, or preservative-free artificial tears can provide temporary relief [10]. Patients with severe LSCD require a series of surgeries, including AMT, conjunctival limbal autograft, conjunctival limbal allograft, and simple limbal epithelial transplantation to reconstruct the ocular surface and restore the stem cell population [8, 10].

It's worth noting that allogeneic transplants differ significantly from autografts. In unilateral LSCD cases, donor tissues are mainly obtained from the unaffected eye, which are called limbal autografts. In the cases of bilateral total LSCD, the donor tissues mainly come from allogeneic source, known as limbal allografts. Both types of surgeries share the common goal of transplanting new epithelial stem cells to reconstruct the ocular surface [9]. At present, the immune rejection is a major risk of allograft failure, so patients need long-term immunosuppression, even in HLA-matched donors, which may result in serious side effects, including hyperglycemia, renal function impairment, anemia and so on [112]. Except for these, a rare case was reported that ocular surface squamous neoplasia was detected in a patient receiving immunosuppression for 3 years after undertaking allografts, which was related to host DNA, indicating the importance of cancer surveillance after receiving allografts [113]. Therefore, how to reduce the side effects of long-term immunosuppression and improve the survival rate of patients is a critical direction of future research.

Collectively, each therapeutic strategy has its advantages and limitations, with various ranges of application and success rates. The following sections provide a brief description of current and emerging techniques for restoring LESCs functionality (Fig. 5).

Amniotic membrane transplantation

The human AM is the innermost layer of the placenta, which can mimic the natural stem cell niche and enhance the self-renewal potential of LESCs [114]. It is considered an ideal substrate for expansion and transplantation of LESCs. Although AM does not provide stem cells, it can promote the proliferation and migration of residual stem cells, help repair the cornea, improve vision, relieve pain and photophobia, and have a significant therapeutic effect in some patients with LSCD [115]. AM also contains a variety of growth factors, protease inhibitors, and anti-inflammatory and anti-angiogenic factors and thus possesses potent anti-angiogenic, anti-inflammatory, and anti-scarring effects [32, 116]. In addition, extracellular vesicles made from human amniotic epithelial cells have also been found to provide an extracellular matrix environment for eye injury repair [117]. Over the past decade, AM has emerged as an ideal substrate for various ocular surface transplantation procedures. However, AM has low transparency and tensile strength and carries the risk of disease transmission, which requires further improvement.

Conjunctival limbal autograft

CLAU is still the first choice for unilateral LSCD because it is harvested from the contralateral eye with no immune rejection and a high transplantation success rate. Previous studies have shown that the surgical success rate of CLAU in the short-term and medium-term follow-up is 80-100%, and the visual acuity is improved by 25-100% [118]. Long-term follow-up showed that after 3 years and 6 years, the success rates were 76% and 62%, respectively [119]. Although CLAU is a reliable and mature surgical method, there remains a risk of contralateral eye injury. Therefore, it is necessary to pay attention to the preoperative evaluation of the contralateral eye to prevent postoperative complications.

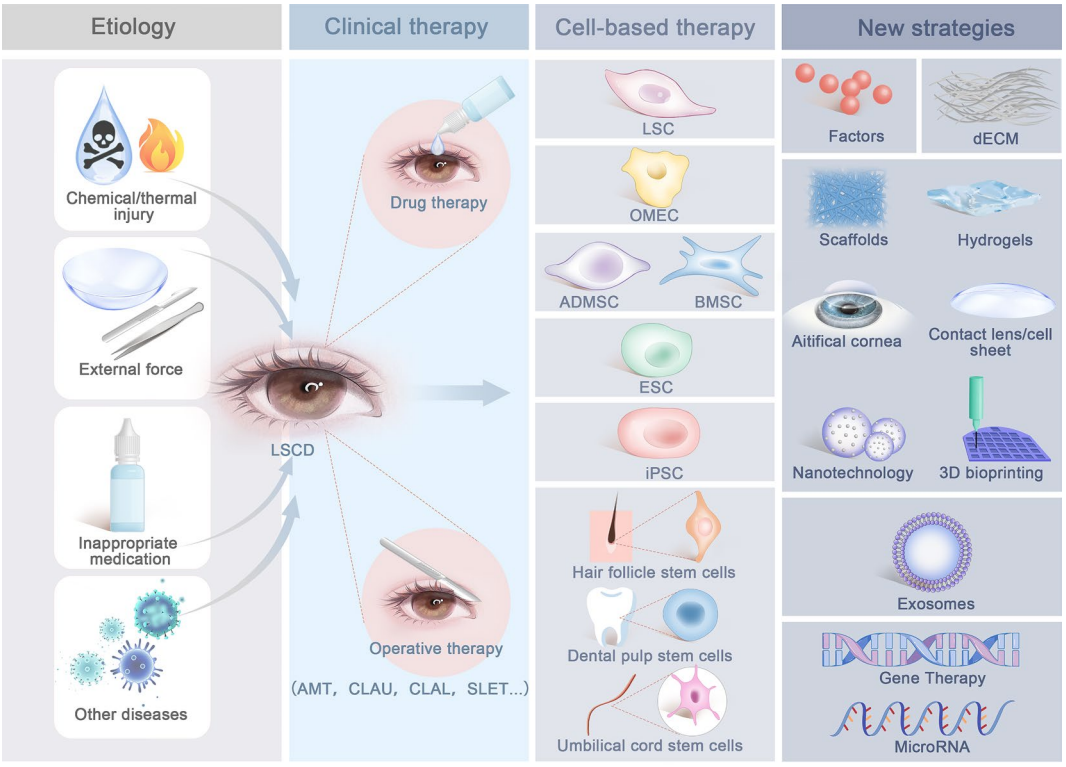


Fig. 5 The brief schematic diagram of current and emerging techniques for treating LSCD

Conjunctival limbal allograft

CLAL transplantation for ocular surface reconstruction is an effective method for patients with bilateral LSCDs. CLAL donor materials can be derived from living relatives or deaths, and plant materials can be harvested from the same material as the CLAU [120]. Cheung et al. [121] conducted a retrospective survey of 63 eyes that underwent CLAL. The results demonstrated that 82.5% patients could maintain stable ocular surface with a mean follow-up time for 7.2 years (range 1.0–16.0 years). Another study reported that the success rate of CLAL ranged from 50 to 100% at final follow-up (range 16–49 months) by evaluating 9 publications, which were affected by the risk of immune response and allograft rejection to some extent [122, 123]. In addition, many eyes receiving allografts have glaucoma due to the initial injury and continue to have or develop high intraocular pressure after surgery, which could also be observed in some patients underwent CLAU [124]. At present, some studies have used histocompatible antigen donor-recipient matching, systemic immune agents, intraoperative use of cyclophosphamide for graft harvesting, and fibrin glue for graft fixation to reduce immunity and rejection reactions [125]. In summary, CLAL remains a viable option for patients with total bilateral LSCD.

Simple limbal epithelial transplantation

SLET is a novel technique for the treatment of unilateral LSCD, first reported by Sangwan et al. in 2012. A 2×2 mm donor limbal tissue was obtained from a healthy eye, divided into 8–15 small pieces, placed evenly on AM adhered to the cornea, and finally covered with a bandage lens on the surface [126]. This technique requires very little limbal tissue and poses minimal risk to the donor's eye. As reported, 68 eyes of 68 patients received SLET in eight centres from three countries, with a clinical success rate of up to 83.8% and a survival rate of more than 80% at a median follow-up of 12 months [127]. More importantly, SLET does not require clinical-grade stem cell laboratory support and has the advantages of low cost and easy replication by corneal surgeons [128]. In a survey of 99 surgeons, involved about 1174 patients who underwent SLET, showing that the cost of undertaking SLET is about 8-10% of the cost of CLET [129]. Recently, a retrospective systematic study, involving 103 eyes in 94 patients with LSCD (mean age 45.0±16.4 years), innovatively compared three epithelial transplantation techniques (SLET, CLET and COMET). The median follow-up results demonstrated that the success rates of SLET, CLET and COMET after 75 months were 77.8%, 45.5% and 57.8%, respectively, and the 7-year survival rates after SLET, CLET and COMET were 72.2%, 50.0% and 53.2%, respectively [130]. Nowadays, SLET tends

to become the preferred technique of limbal stem cell transplantation.

However, there are still some complications, such as further progression of corneal conjunctivalisation, symblepharon, aseptic or bacterial keratitis, recurrence of corneal neovascularization, persistent corneal epithelial defect, and corneal epithelial hyperplasia [131]. Therefore, more clinical trials should be conducted and the long-term effects need to be further determined.

Cell-based therapy

Despite the modest success of these procedures, graft rejection-related issues and the limited availability of suitable donors are major obstacles to successful LESC transplantation. In recent years, stem cell-based therapies have moved to the forefront of regenerative medicine. In addition, other sources of stem cells, such as oral mucosal epithelial cells, mesenchymal stem cells, human embryonic stem cells, pluripotent stem cells, hair follicles, and dental pulp stem cells, have great potential for corneal epithelial regeneration after transplantation [132].

Cultured limbal epithelial transplantation (CLET)

Pellegrini et al. [133] pioneered clinical trials using cultured autologous limbal epithelial cells for transplantation. After more than 10 years of clinical follow-up, corneal regeneration can be achieved in more than 70% of cases. CLET requires a small number of donor cells, which minimises the risk to the donor eye and reduces the risk of LSCD in the donor eye [134]. Surprisingly, CLET results appeared to be similar no matter whether the cell source was autologous or allogeneic. Zhao et al. [135] analyzed 18 publications (involving 572 eyes, 562 patients) and found that the success rates of autologous CLET and allogeneic CLET (receiving systemic immunosuppression) were both about 67%, which were similar with another recent meta-analysis of autologous CLET (982 eyes) and allogeneic CLET (324 eyes) conducted by Mishan et al. [136]. However, due to the patient's individual differences and various evaluation index, more clinical trials should be conducted to further validated. Currently, in the United States, clinical trials (NCT03957954 and NCT02592330) are investigating the safety and feasibility of cultured autologous LSCs for treating LSCD [137]. More encouragingly, the first and only stem cell therapy for autologous LSCD (trade name, Holoclar) has received conditional marketing authorisation in the European Union [138].

Although the overall success rate of CLET is reported to be between 50% and 85%, the current culture techniques of isolated cells still need strict sterile environment, advanced hardware facilities, and high costs [110]. In addition, sub-graft haemorrhage, infectious keratitis, rejection, glaucoma, persistent epithelial defects, corneal perforation, and cyclosporin-related adverse reactions may occur [139]. These factors limit the clinical use of CLET.

Cultivated oral mucosal epithelial transplantation (COMET)

To solve the problem of donor tissue shortage, studies have been conducted to identify other sources of stem cells for ocular surface reconstruction, among which transplantation of oral mucosal epithelium cultured in vitro has been shown to be a safe and effective alternative, with a success rate as high as 80% [140]. Oral cells transplanted onto the surface of the cornea can survive and steadily undergo ocular reconstruction, expressing the proliferation marker Ki67, progenitor marker p63, and corneal epithelial markers CK3 and CK12, without long-term systemic immunosuppression. COMET has a significantly higher angiogenic potential than cultured limbal epithelial cells and no tumourigenic events have been reported [141]. Ocural®, the world's first in vitro cultured oral mucosal epithelial cell transplantation for the treatment of LSCD, was launched in Japan in June 2021 [142].

Failure of in vitro COMET is primarily related to persistent corneal epithelial defects, corneal surface neovascularization, and corneal surface fibrosis. Nowadays, the mechanisms underlying the transformation of oral mucosal epithelial cells into differentiated corneal epithelial cells are still poorly understood [143].

Mesenchymal stem cells

MSCs are multipotent mesenchymal stromal cells similar to fibroblasts. Compared with limbal epithelial cells, MSCs have many potential advantages: (1) MSCs can be obtained from many tissue types, including the bone marrow, adipose tissue, and umbilical cord; (2) MSCs can be cultured in vitro and reach the clinical scale in a short time using less expensive procedures than LESCs. (3) High plasticity, strong self-renewal capability, satisfactory immune regulation, and anti-inflammatory ability through the secretion of numerous cytokines [46, 144].

Studies have shown that cultured limbal MSCs express stem cell markers similar to LESCs, such as p63α, PAX6, ABCG2, and ABCB5, suggesting that limbal derived mesenchymal stem cells have excellent plasticity [145]. Notably, human limbal-derived MSCs completely prevented interstitial scar formation in a mouse corneal wound model when human MSCs were injected into mouse corneas [146]. A clinical trial testing the ability of MSCs to reverse interstitial scarring in human is currently underway (NCT02948023).

Bone marrow-derived MSCs (BM-MSCs) are also a promising therapeutic option. The first clinical use of MSCs for LSCD was reported in 2019 by Calonge et al. [146], in which 22 patients with severe and complete

LSCD were randomised to receive either allogeneic bone marrow mesenchymal stem cell transplantation (MSCT) or CLET. After one year, the success rates of MSCT and CLET were 85.7% and 77.8%, respectively, and no adverse events occurred. However, these results need to be confirmed in a larger number of patients. Adipose tissue is also a rich source of MSCs, with the advantages of simple collection, high proportion of stem cells, and low risks of liposuction complications [147]. Several studies have shown that human adipose-derived mesenchymal stem cells (ADSCs) promoted the proliferation of LESCs through paracrine activity. The application of ADSCs in the LSCD rat model significantly improved the function of promoting corneal wound repair, restored transparency, and regulated paracrine effects [148]. ADSCs can also accelerate the clearance of corneal neutrophils, inhibit corneal neovascularization, and promote corneal wound healing [149]. One study [150] innovatively combined insulin-like growth factor 1 (IGF-1)-modified mRNA technology with ADSCs therapy ($ADSC^{modIGF1}$) for alkali-burned corneas in mice. Compared with ADSCs alone and IGF-1 protein eye drops, $ADSC^{modIGF1}$ could more effectively inhibit the formation of blood and lymphatic vessels and promote healing of the corneal epithelium (Fig. 6). Moreover, it could significantly promote the activity of trigeminal ganglion cells and maintain the stemness of human LESCs, which were necessary for reestablishing corneal homeostasis.

Currently, there are several ways to transfer MSCs, including local administration, subconjunctival administration, and bone and interstitial injections [151, 152]. However, there is no consensus regarding the optimal route for MSCs delivery. Further studies are required to determine suitable delivery systems and understand the mechanisms underlying their therapeutic properties.

Human embryonic stem cells

HESCs are pluripotent stem cells derived from a population of cells within the human blastocyst that can differentiate into derivatives of all three germ layers. Importantly, hESCs can differentiate into corneal or limbal epithelial cells, providing an unlimited source of cells for the treatment of patients [153].

A stem cell environment that induces the differentiation of hESCs into corneal or limbal epithelial-like phenotypes is very important. For example, Martins et al. [154] innovatively used the acellular corneal epithelial basement membrane as a substrate to induce embryonic stem cells to differentiate into corneal epithelial-like cells. Zhang et al. [155] successfully induced hESCs into corneal epithelial progenitors using serum-free media and transplanted them into rabbit eyes. Recently, He et al. [156] also proved that sheets of clinical-grade hESC-derived corneal epithelial cells successfully repaired damaged ocular surfaces in rabbit LSCD models. These results further highlighted the potential of hESCs as a limited source of cells.

However, these differentiation techniques rely on culturing hESCs on corneal tissue donors or preparing conditioned media. Due to their embryonic origin, difficulty in differentiating into pure cultures, immunogenicity, ethical issues, and potential tumourigenicity, there is still a long way to go before consistent clinical application of hESCs in treating LSCD [157].

Induced pluripotent stem cells (iPSCs)

Human-induced pluripotent stem cells (hiPSCs) are generated by manipulating differentiated adult cells and have properties similar to those of hESCs. Compared to other stem cell sources, iPSCs are easily scalable and have advantages in terms of differentiation potential and ethical issues; therefore, they are theoretically considered an

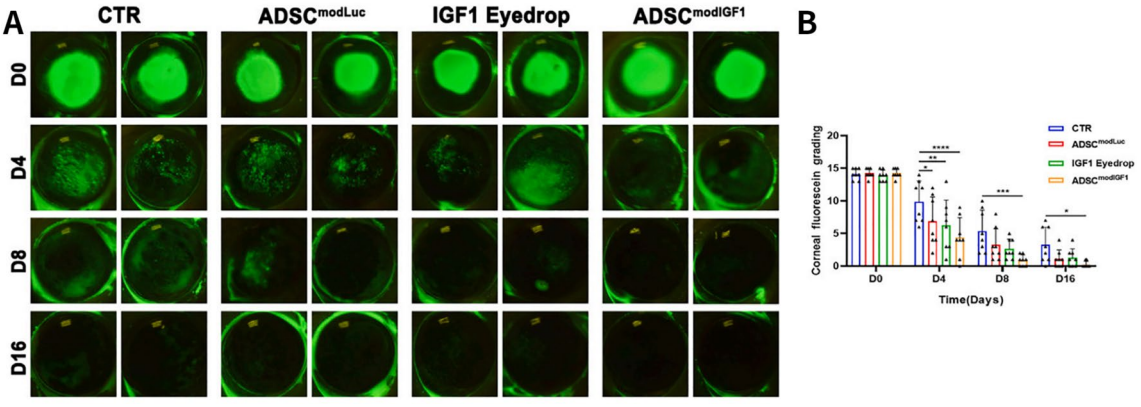


Fig. 6 The fluorescein staining images of corneal epithelial healing. (**A**) Representative fluorescein staining images of corneal epithelial healing in each group at 0, 4, 8, and 16 days after alkali burn. (**B**) Groups of corneal fluorescein score analysis and comparison. Reproduced with permission. Copyright 2023, Molecular Therapy

unlimited source of renewable cells to meet future transplantation needs [158].

Hayashi et al. [159] reported the first method for extracting hiPSCs from adult limbal epithelial cells and human dermal fibroblasts to generate corneal epithelial cells. Subsequently, corneal stem cells and progenitor cells were generated from hiPSCs by replicating eye development in vitro, and an epithelial cell sheet was generated, successfully restoring corneal function in rabbit LSCD models [160, 161]. Interestingly, Susaimanickam et al. [162] used human-induced pluripotent stem cells to generate corneal organoids that mimic corneal development, which held promise for the development of predictive diagnostic markers, drug testing, and personalised medicine.

In summary, advances in iPSC technology, along with gene editing and bioengineering, have provided opportunities for iPSC applications in corneal development, disease modelling, drug discovery, and regenerative medicine. However, its high cost, low conversion rate, risk of tumour formation, and potentially unpredictable biological changes hinder its clinical application [163]. Currently, iPSCs-derived LESCs are used in Phase I clinical trials (JPRNUMIN000036539), and further improvements are being made to the derivative regimen to achieve a transition from the laboratory to bedside as soon as possible.

Stem cells from other sources

In addition to the aforementioned stem cells, hair follicles, dental pulp, and umbilical cord stem cells are excellent candidates as stem cell sources. Blazejewska et al. reported that hair follicle stem cells could effectively differentiate into corneal epithelial-like cells in the culture medium of limbus fibroblasts, and an 80% success rate of trans-differentiation was observed in a mouse LSCD model [164]. Dental pulp stem cells expressed cytokeratin specific to the corneal upper cortex; therefore, they were considered an ophthalmic treatment option [165]. In a rabbit LSCD model, grafts containing human immature pulp stem cells were transplanted into the limbic niche. After three months, LESCs markers were detected in human immature pulp stem cells, and the ocular surface condition improved [166]. Human umbilical cord intimal epithelial cells are another potential source for treating LSCD, and animal models using these stem cells are available [167]. However, further studies are required to generalize these potential sources to humans.

Recent advances in LSCD

With the progress of science and technology, new biotechnology strategies for LSCD have been tested in recent years, such as exogenous factors, decellularized matrix, tissue engineering, nanocarriers, exosomes, gene therapy and miRNAs, which can bring hope to patients with LSCD.

Exogenous factors

Local administration of exogenous growth factors has been proposed as a non-invasive approach to restore marginal niche function. Many exogenous factors, such as epidermal growth factor (EGF), pigment epithelium-derived factor (PEDF), basic fibroblast growth factor (bFGF), IGF1, and vitamin A, are very important for the regeneration of the limbal niche, which may help patients with LSCD recover ocular surface health [168].

Baradaran-Rafii et al. reported the regenerative effect of AM extract eye drops on LESCs in patients with LSCD [169]. Similarly, Chen et al. [170] extracted HC-HA/PTX3 from the AM to restore senescent limbal niche cells to $Pax6^{+}$ neural crest progenitor cells by activating CXCR4 and BMP signaling to support the self-renewal of limbal epithelial progenitor cells. IL13 enhanced the stemness of human LESCs by increasing clonogenicity and the expression of putative stem cell markers [171]. Lee et al. [172] found BMP4 could induce hiPSCs to the progenitor cells of limbus of cornea, and 10 ng/mL BMP4 for 3 days is the best scheme to maintain the phenotype of human LESCs. These findings will contribute to the development of novel therapies for LSCD.

Despite the reported beneficial effects of these drugs and growth factors, their efficacy in LSCD remains limited, and further studies are needed to provide more inspiring growth factors as potential therapeutic options for LSCD to activate and preserve the remaining LESCs.

Decellularized matrix

The extracellular matrix is not only a supporting scaffold but can also simulate the cellular microenvironment and regulate the behaviour of cells through direct and indirect signal transduction. Therefore, supplementing the ECM constructed by tissue regeneration may be a viable technique for restoring the role of marginal niches. Decellularized native tissues have the advantage of faithfully replicating the native ECM, including the decellularization of human cornea, collagen, and animal proteins [173].

Wang et al. [174] prepared acellular porcine corneal stroma using phospholipase A2 decellularization and crosslinked it with aspartic acid. Compared to the control group, rabbit LESCs showed a three-dimensional cell sphere structure and improved stem cell performance. In the rabbit lamellar keratoplasty model, the reconstructed auto-tissue engineering lamellar cornea (ATELC) quickly recovered the natural optical properties within 1 week after transplantation, and the nerve regeneration and interstitial regeneration were good at 6 months after keratoplasty, demonstrating the great potential of ATELC as

a corneal substitute for future applications (Fig. 7). Yazdanpanah et al. [175] reported the potential regenerative effect of an eye-bandage hydrogel made from decellularized porcine corneal ECM in a mouse corneal epithelial wound healing model, thus providing a promising option for patients with LSCD.

Similarly, Shen et al. [176] designed a hybrid hydrogel consisting of porcine decellularised corneal stroma matrix (pDCSM) and hyaluronic acid methacrylate by a non-competitive double crosslinking process. Mixed hydrogel not only retained the bioactive components of pDCSM but also supported the viability and proliferation of corneal cells to accelerate corneal re-epithelialisation with 37 ± 4 μm thickness and wound healing for 8 weeks, which can be effectively used for long-term wireless suture and tissue regeneration after corneal defect.

Notably, the light-curable corneal matrix (lc-commatrix) derived from acellular porcine corneal ECM could be used as a functionalized hydrogel and enhanced its biomechanical strength, stability, and adhesion to the human cornea. In vivo, lc-commatrix could seal large corneal perforations, replace part of corneal stroma defects with 397 ± 12 μm central corneal thickness at last follow-up, and bio-integrate into tissues in a rabbit model after 28 days, which demonstrated its potential application prospect in corneal and LSCD ophthalmic surgery [177].

These studies suggested that decellularized tissues with native ECM scaffolds could not only play an important role in the regulation of stem cells, but also induce stem cells to differentiate into the cell types present in certain tissues. Nowadays, decellularized organs have been used in tissue engineering and cell therapy.

Tissue engineering

Scaffolds

At present, a variety of natural (such as AM, collagen, and fibrin) and synthetic polymer materials have been developed as carriers for LESCs, and a number of preclinical studies have been conducted [108]. Wang et al. [178] innovatively used the onion epithelial membrane (OEM) as a carrier to expand rabbit corneal epithelial cells and treat corneal epithelial defects in rabbits with LSCD. The results showed that OEM promoted corneal epithelial wound healing, shortened the time required for wound healing, and formed a more compact and stratified epithelial framework than in the untreated group.

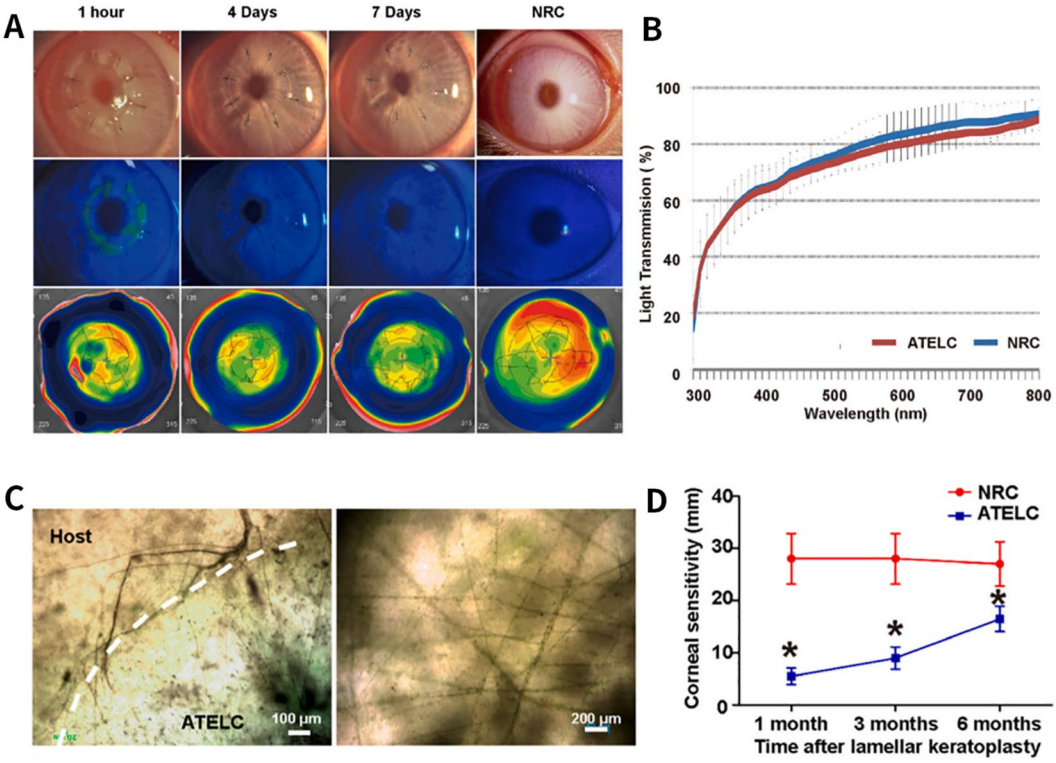


Fig. 7 The repair of the corneal epithelium and nerves. (**A**) Transparency, sodium fluorescein staining, and corneal topography results in normal control cornea (NRC) groups and ATELC at 1 h, 4 days, and 7 days after keratoplasty. (**B**) Transmittance in the 300–800 nm wavelength range 7 days after keratoplasty. (**C**) Corneal nerve staining images of ATELC group rabbits at 6 months after keratoplasty. (**D**) The results of corneal mechanical sensitivity in NRC group and ATELC group at 1 month, 3 months and 6 months after keratoplasty. *$p < 0.05$ ($n = 10$). Reproduced with permission. Copyright 2022, Biomaterials

These findings highlighted that plant-derived implants might be an attractive option for clinical practice.

Synthetic polymer scaffolds, such as polycaprolactone (PCL), polylactic acid (PLA), and polylactic co-glycolic acid, are attractive alternative substrates. They have unique advantages in design, material sources, standardised mass production, and operability [179]. Zdraveva et al. [180] designed electrospun PCL coated with anti-VEGF, which increased the fibre diameter and pore area by ~24% and 82%, respectively. The results confirmed that PCL/anti-VEGF not only had good biocompatibility but also facilitated the adhesion, growth, and differentiation of human LESCs, with the expression of p63 and CK3 markers. Ramachandran et al. [181] evaluated the safety and efficacy of PLGA scaffolds for regenerating LESCs in simple limbal epithelial grafts in five patients with LSCD. In all five patients, the epithelium regrew after 3 months and 60% of the subjects had a clear ocular surface with no epithelial defects at 12 months. In the future, more functional scaffolds will be developed for the treatment of LSCD.

Hydrogels

Hydrogels are hydrophilic molecules with three-dimensional networks that exhibit good biocompatibility, optical properties, and adjustable mechanical behaviour [182]. Hydrogel-based stem cell therapy is a popular approach for the treatment of LSCD. Zhong et al. [183] used digital light-processing-based bioprinting to fabricate engineered microscale hydrogel scaffolds based on gelatin methacrylate (GelMA) or hyaluronic acid glycidyl methacrylate (HAGM). These scaffolds not only supported the viability of encapsulated primary rabbit LESCs and hLESCs, with 86.7±1.6% live cell ratios in GelMA scaffolds and 92.1±0.8% in HAGM scaffolds after 7 days, but also showed differential regulation innovatively. Interestingly, LESCs proliferated actively in GelMA-based scaffolds but presented a quiescent state in HAGM-based scaffolds for six days. Therefore, a novel bio-printed dual ECM "Yin-Yang" model that encapsulated LESCs to support both active and quiescent states was developed, which provided valuable insights into stem cell therapy in LSCD.

Recently, Shi et al. [184] compounded electrospun nanofibres of thioketal-containing polyurethane (PUTK) with a ROS-scavenging hydrogel (RH) to produce PUTK/RH patches with good transparency, hydropathy, and antioxidant capacity. In a rat corneal alkaline burn model, the PUTK/RH patch accelerated corneal wound healing by inhibiting inflammation, promoting epithelial regeneration, and reducing scar formation. On day 7, the areas of opacity in the PUTK/RH group (24.5±8.9%) were significantly smaller than that in the alkali-burn group (37.9±8.0%), suggesting that hydrogels could be used in various forms for ocular surface reconstruction.

Nowadays, the development of wireless suture technology is a direction for future research. Koivusalo et al. [185] partially grafted dopamine onto hydrazone-crosslinked hyaluronic acid (HA-DOPA) hydrogels to develop a tissue-adhesive scaffold with hESC-LESCs on the surface and encapsulated human adipose-derived stem cells (hASCs) in the hydrogel bulk, exhibiting good proliferation and cell elongation for 2 weeks. Importantly, the hydrogels showed high swelling ratios and high adhesion forces on the ocular surface (Fig. 8). These results encouraged the sutureless implantation of functional stem cells as the next generation of corneal regeneration and laid a profound foundation for hydrogels in LSCD and corneal epithelial repair.

Artificial cornea, epithelial cell sheet and contact lens

The development of artificial corneas has provided hope for the treatment of LSCD. Boston KPro (Dohlman-Doane keratoprosthesis) is an FDA-approved fabrication method and is the gold standard for keratoprosthesis [186]. In addition, numerous synthetic materials such as poly (2-hydroxyethyl methacrylate) (PHEMA), polyethylene (glycol) Diacrylate (PEGDA), poly (methyl methacrylate) (PMMA), poly (vinyl alcohol) (PVA) and PLGA have been used for corneal bioengineering [187]. Bolagh et al. [188] developed synthetic corneas dominated by protein elastomers by tightly binding bioactive dual elastins to mechanically robust protein filaments. They have properties similar to those of natural corneas in terms of optical clarity, refractive index, glucose permeability, and mechanical properties, and support the growth and function of corneal epithelial and endothelial cells. Recently, Hao et al. [189] designed bioartificial corneas (BACs) according to the standards for Class III medical devices. Mechanical strength was improved by self-crosslinking aldehyde-modified hyaluronic acid and carboxymethyl chitosan on the surface of decellularised porcine corneas without the use of crosslinking agents. BACs not only had good biocompatibility, transparency, and anti-inflammatory properties, but also can rapidly regenerates the epithelium and restores vision within one month. After 3 months, the BACs were gradually filled with epithelial, mesenchymal, and neuronal cells, and after 6 months, the BACs were essentially normal in clarity and histology. This product can be used as a substitute for corneal reconstruction in vitro to address donor corneal insufficiency. AiNear" is the first and only commercially available bioengineered corneal product worldwide.

Besides, the in vitro expansion of autologous and allogeneic LESCs into epithelial cell sheets is a novel strategy for treating LSCD. One study [186] performed a clinical trial of autologous cultured human limbal epithelial cell

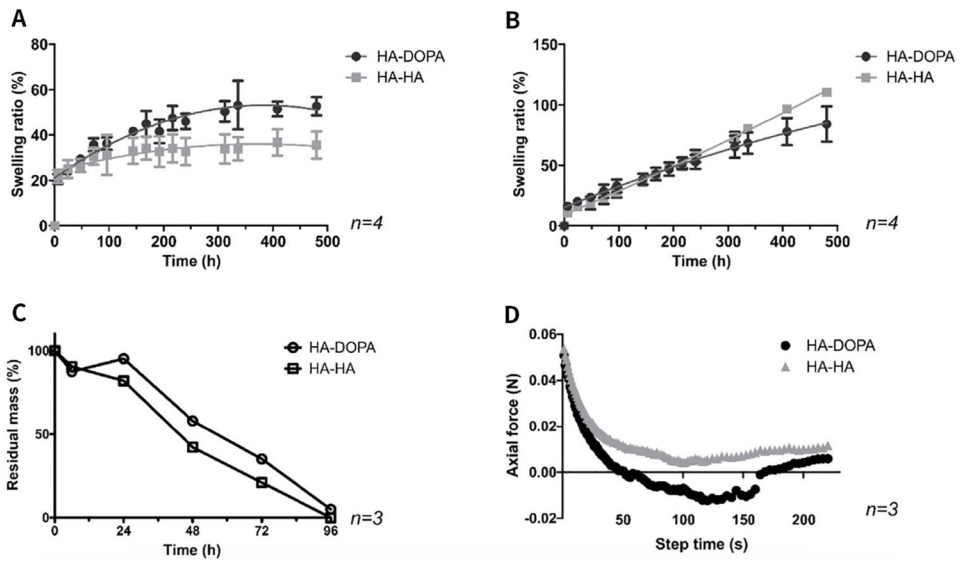


Fig. 8 The properties and characterization of hydrogels (**A**) The measurement of hydrogel swelling ratios in PBS. (**B**) The measurement of hydrogel swelling ratios in cell culture medium. (**C**) Enzymatic degradation of the hydrogels in the presence of hyaluronidase. (**D**) Adhesion force of the hydrogels to corneal surface. Reproduced with permission. Copyright 2019, Biomaterials

sheet transplantation for ocular repair in 10 eyes of 10 patients with unilateral LSCD. The ocular reconstruction rate was 70% at 2 years after surgery. In addition, vision improved 50% and 60% of eyes at 1 and 2 years, respectively. No clinically significant transplantation-related adverse events were observed. Thus, the efficacy and safety of transplantation of cultured marginal epithelial cell plates were confirmed. This cell plate, named " Nepic " is now recognised as a cell- and tissue-based product in Japan.

Contact lenses have many advantages, such as ease of use, convenient drug loading, and broad application prospects, in the treatment of eye diseases. Kushnerev et al. [190] used soft contact lenses to deliver in vitro expanded human dental pulp stem cells to promote corneal epithelial regeneration and reduce corneal conjunctivalisation. In a retrospective study of a consecutive series of 267 LSCD patients using scleral lenses, Bonnet et al. found that scleral lenses improved corrected distance visual acuity, although LSCD still progressed in some patients [191]. Unfortunately, prolonged or incorrect contact lens use can cause a series of complications, such as eye discomfort, dry eye, corneal abrasion, corneal neovascularization, and even further deterioration [192]. Reducing such complications and improving the treatment effects are directions for future research.

Nanotechnology

Nanotechnology has played an important role in the treatment of ophthalmic diseases, with the advantages of overcoming ocular barriers, prolonging drug retention time, reducing drug delivery frequency, and improving patient compliance [193]. Nano-based delivery of drugs, genes, and miRNA-siRNAs to specific cells, including LESCs, has been used for the treatment of LSCD.

Biopolymer bacterial nanocellulose (BNC) is synthesised from non-pathogenic bacterial cultures in the form of a staggered nanofibre structure that shows good stability and immunogenicity [194]. Anton-Sales et al. [195] used the BNC to grow hESC-derived LSC. BNC not only support LSCs to maintain self-renewal and stemness for up to 21 days but also provide independent and easy mechanical support (Fig. 9). After the functionalization of plasma-activated ECM proteins on BNC substrates, the attachment and viability of hESC-LSCs were enhanced without compromising the flexibility, robustness, and translucency properties of BNC.

Zhou et al. [196] constructed a composite membrane consisting of electrospun PCL nanofibres with acellular AM. The ultimate tensile strength, toughness, and suture-holding strength were 4–10 folds improved compared to decellularised AM, along with greater stability and longer-lasting corneal surface coverage. Importantly, in the rabbit LSCD corneal epithelial defect model, the composite membrane maintained the pro-regenerative and immunomodulatory properties of decellularised AM; promoted the survival, retention, and organisation of LESCs; improved re-epithelialisation of the defect area; and reduced inflammation and neovascularization. This study demonstrated the translational potential of composite membranes in stem cell therapy for ocular surface injuries. In summary, nanotechnology has broad application prospects for the treatment of corneal diseases.

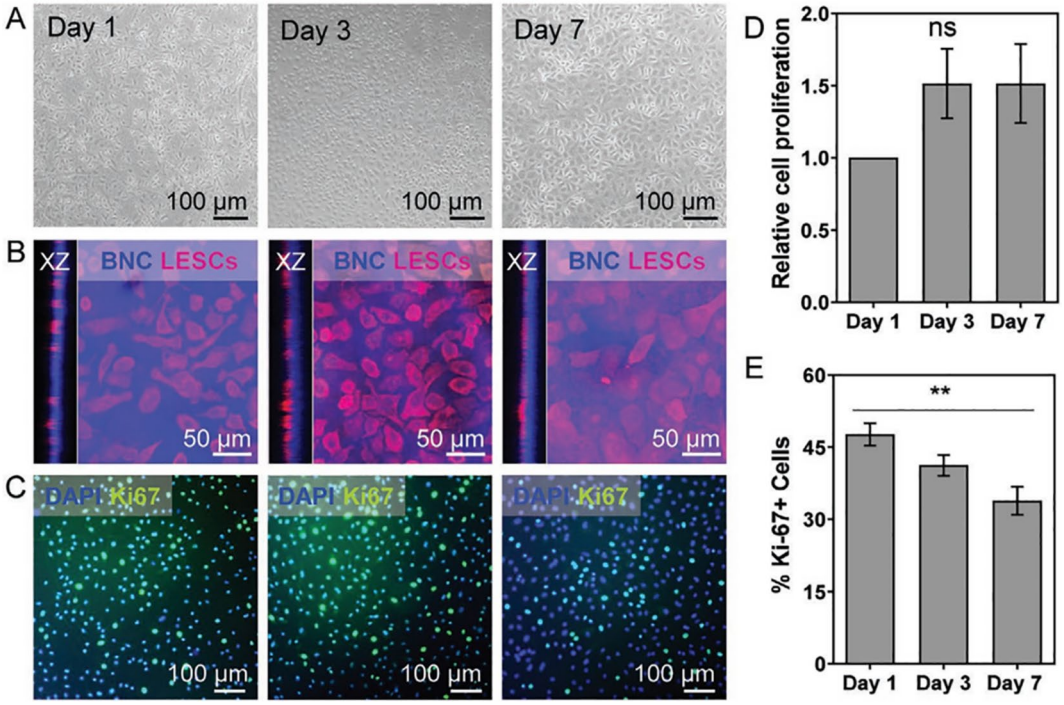


Fig. 9 The culture monitoring and proliferation of hESC-LSC on BNC. (**A**) The distribution and cell density of hESC-LSC on BNC substrates. (**B**) Confocal microscopy and XZ section images showing the tendency of hESC-LSC to become an epithelial monolayer on BNC substrate. (**C**) Representative immunofluorescence staining image of Ki67 proliferation marker expression. (**D**) The metabolic activity of hESC-LSC. (**E**) Analysis of the percentage of Ki67-positive cells at three different culture time points. Reproduced with permission. Copyright 2021, Small

3D bioprinting

Using 3D bioprinting to design innovative biomaterials can help stem cells form a more compatible biomimetic niche, improve their integration into the cornea, minimise cell death, and prevent side effects [197].

Dehghani et al. [198] prepared 3D printed membranes using a mixture of gelatin (8% w/v), elastin (2% w/v), and sodium hyaluronate (0.5% w/v), with good optical properties and biocompatibility. Compared to that in the AM group, twice the density of goblet cells per 100 cells and lower levels of clinical inflammation from day 1 to day 28 was observed in the membrane-grafted group, which is a key advantage of 3D printed membranes. Sorkio et al. [199] used laser-assisted 3D bioprinting and a functional bioink to simulate the tissue structure of human corneal stem cells. Laser-printed hESC-LESCs showed epithelial morphology, Ki67 proliferation marker expression, and co-expression of the corneal progenitor cell markers p63a and p40. In addition, the hASCs displayed a collagen-positive marker and showed signs of hASCs migrating from the printed structures after 7 days of porcine organ culture. These results further demonstrated the importance of 3D printing and functional bioinks in ocular surface reconstruction.

Exosomes

Exos are natural lipid bilayer vesicles approximately 40–150 nm in size that belong to the extracellular vesicle (EV) family. Their biological regulation ability, cell-free state, and long-term storage make exosomes potential delivery vehicles for the therapy of corneal diseases [200]. For instance, Leszczynska et al. [201] concluded that limbal keratinocyte Exos from healthy and diabetic patients have different efficiencies in regulating the migration and proliferation of LESCs. In addition, topical MSC-Exos can reduce corneal damage by promoting wound healing and reducing scar development through anti-angiogenesis and immune regulation [202].

Many exosome functions are mediated by their encapsulated miRNAs and vesicular contents (lipids, proteins, and nucleic acids) that target cells through paracrine signaling and microenvironmental modifications. Studies have shown [203] that corneal stromal stem cell-derived extracellular vesicles (CSSC-EVs) could promote human LESC proliferation and stem cell maintenance by targeting the Notch signaling pathway through miRNAs, which can be used as a supplement to the culture medium to expand the LESC population. In addition, the regenerative, immunomodulatory, and anti-vascularisation properties of CSSC-EVs provide new research directions for LESC transplantation and other corneal epithelial diseases [204].

The combination of exosomes and other materials represents a new developmental direction. Topical treatment of the corneal surface with nanopolymers or exosomes loaded with c-Rel-specific siRNA can effectively accelerate conventional and diabetic corneal wound healing [205]. Tang et al. [206] developed a thermosensitive chitosan-based hydrogel with a sustained release of iPSC-MSC exosomes. iPSC-MSCs inhibited the mechanism related to the target gene TRAM2 by secreting exosomes containing miR-432-5p to prevent ECM deposition and promote the repair of damaged corneal epithelium and stromal layers, which opens new avenues for the clinical utility of exosome-loaded thermosensitive hydrogels. Sun et al. [207] combined the advantages of Exos (anti-inflammatory, cell proliferation, and migration) and miRNA 24-3p (cell migration) to prepare miRNA 24-3p-rich exosomes (Exos-miRNA 24-3p) for corneal epithelial injury repair. In addition, a heat-sensitive di (ethylene glycol) monomethyl ether methacrylate-modified hyaluronic acid hydrogel was developed to control the release of Exos-miRNA 24-3p in a rabbit alkali-burn model. Exo-miRNA 24-3p effectively promoted the migration and maturation of rabbit corneal epithelial cells and corneal tissue repair 28 days after the alkali burn. This study provided a promising miRNA-based multilevel delivery strategy for the efficient and adaptive treatment of corneal alkali burns and provides an important theoretical basis for the development of cell-free therapy.

Currently, exosomes are the most promising transport carriers and have been commercialised by several biological companies [208, 209]. However, the extraction of exosomes requires high technical requirements and a large amount of cost. In addition, the role of exosomes in ocular pathophysiology remains unclear. At the same time, there are concerns regarding the ability of exosomes to cross the human corneal epithelial barrier because most studies were conducted in animals.

Gene therapy and microRNA

Gene therapy has great potential for treating human corneal diseases. The immune-privileged nature and availability of the cornea make it an important target for gene therapy [210]. The reagents of genes can be locally applied to the corneal surface and visually monitored by labelling target genes with fluorescent proteins. Gene therapy for wound healing, as well as for miRNAs, is emerging as an important controller of stem cell potency, proliferation, and differentiation [211].

Recently, Ali et al. [212] reported lentiviral vector (LV)-mediated gene delivery to LESCs following corneal injection in mice. In this case, gene expression persisted in the corneal epithelial cells for one year, which was highly suggestive of permanent LESC gene modification. In addition, Valdivia et al. [213] used LV encoding short hairpin RNAs to silence the expression of human leukocyte antigens, which not only maintained the typical morphology, phenotype, and proliferation characteristics of LSCs in vitro but also reduced the allogeneic immune reaction. Song et al. [214] reported for the first time that Adeno-associated viral 6 and LV successfully delivered genes to human primary limbal epithelial cells or LESC colonies. Stable transgene expression was observed after LV transduction, highlighting its potential use in the treatment of LSCD.

MiRNAs are endogenous, small, non-coding oligonucleotides with a length of approximately 19 to 25 nucleotides [215]. Some of these are differentially expressed between the limbus and central cornea, which may play an important role in the niche regulation of LESCs. For example, miR103/107 was reported to preferentially express in limbal epithelium and regulate MAP3K7 signaling and JNK activation by targeting NEDD9 (HEF1) and non-canonical Wnt signaling to regulate LESC proliferation and interaction [216]. Has-miR-143-3p was involved in the maintenance of human corneal epithelial stem cell stemness by inhibiting Wnt and MAPK signaling pathways [217]. Similarly, Hsa-miR-150-5p maintained the stemness of human LESCs by inhibiting the Wnt-β-catenin pathway [218]. Additionally, miRNA-146a regulated corneal regeneration and maintained human LESCs stemness. Its overexpression could alter the normal repair functions of diabetic corneas [219]. MiR-145, miR-10b, hsa-miR-10a-5p, hsa-miR-1910-5p, and hsa-miR-21-5p were also highly expressed in human corneal epithelial stem cells [220, 221].

In summary, miRNAs play important roles as natural and powerful regulators of gene expression during corneal epithelial regeneration and tissue repair. The introduction of gene therapy and miRNAs to activate and preserve the remaining LESCs in patients with LSCD is a promising strategy.

Recent ongoing clinical trials for treating LSCD

With the understanding of the LESCs and its regulation, more and more clinical trials are conducted for the treatment of LSCD. In Table 1, we summarized some of the ongoing and completed clinical trials. It is believed that in the future, with the joint efforts of researchers and clinicians, more and more studies can be carried out to reconstruct the stem cell microenvironment, stimulate tissue regeneration and restore the native corneal function, so as to better achieve clinical translation.

Challenges from basic research stages to clinical therapies

Despite researchers achieving a great deal in the field of LESCs, many challenges remain. The first step in studying limbal niche is determining the exact location of

Table 1 Representative clinical trials for treating LSCD

Brief description	Sources	Indications	Trials	Phase
Non-xenogenic limbal stem cell grafts transplantation	LSC	LSCD	NCT02318485	II
Multinational follow-up study of the autologous cultivated LSC transplantation	LSC	LSCD	NCT03288844	NA
Stem Cell Therapy for Limbal Stem Cell Deficiency	LSC	LSCD	NCT03957954	I
Transplantation of cultivated autologous LESC for the affected eye	LESC	LSCD	NCT02592330	I/II
A Multicenter Trial for LESC Transplantation	LESC	LSCD	NCT02318485	II
Autologous Cultured Corneal Epithelium for treating corneal lesions associated with LSCD	Cultured corneal epithelium	LSCD	NCT01756365	NA
		LSCD	NCT03217487	NA
Corneal Epithelial Autograft for LSCD	Corneal epithelium	LSCD	NCT03884569	NA
Observational study of CLET	Limbal epithelium	LSCD due to ocular burn	NCT02577861	NA
Efficacy and Safety of Autologous CLET for Restoration of Corneal Epithelium	Holoclar			
LSCs cultured on AM in treating total limbal deficiency	Cultivated LSC	Total limbal deficiency	NCT01619189	II
The test of ex vivo cultured LSCs on AM for LSCD	Cultured LSCs	LSCD	NCT00736307	II
Amniotic Membrane Extract Eye	Amniotic Membrane	LSCD	NCT02649621	I
Drop for LSCD	Unaffected eye	LSCD	NCT04021134	NA
Observational study of allogeneic SLET	Unaffected eye	LSCD	NCT04021875	NA
Autologous SLET	Mucosal Epithelium	LSCD	NCT03943797	I
COMET				
Transplantation of autologous labial mucosal epithelium as a substitute for LSCs	Mucosal epithelium	LSCD	NCT04995926	NA
Transplantation of Autologous Oral Mucosal Epithelial Sheets for LSCD	Mucosal epithelium	LSCD	NCT02415218	I/ II
The efficacy and safety of cultivated oral mucosal epithelial sheet transplantation for LSCD	Mucosal epithelium	LSCD	NCT02415218	II
	Oral Mucosa	Bilateral LSCD	NCT03949881	I/ II
Autologous Oral Mucosa Epithelial Sheet for Bilateral LSCD	Oral Mucosa	LSCD	NCT03226015	NA
Autologous Oral Mucosa Transplantation with clinical and histochemical results				
COMET	Oral Mucosa	LSCD	NCT02149732	NA
Allogeneic ABCB5-positive Limbal Stem Cells for Treatment of LSCD	$ABCB5^+$ LSC	LSCD	NCT03549299	I/ II
Trials to explore the suitable conditions of corneal epithelial graft culture	Cultured LSCs	LSCD	NCT01237600	III
Pharmacological investigation of nerve growth factor in treating of LSCD along with neurotrophic cornea	Nerve growth factor	LSCD	NCT04552730	NA

LESCs before they can be accurately tracked. Although LESCs preferentially express several markers, no specific molecular markers have been identified.

Recently, the International LSCD Working Group proposed a global consensus to better understand, classify, diagnose, and manage LSCD [10]. However, clinicians have yet to fully agree, and the diagnosis, grading, and outcomes of LSCD are often difficult to interpret for several reasons: (1) the stage, severity, and type of LSCD are poorly defined; (2) the heterogeneity of LSCD aetiology, laterality, graft type, different culture techniques and carriers, various surgical techniques, and subsequent surgical procedures; and (3) the definition of clinical success is unclear.

Although surgical treatments and stem cells from various sources have achieved remarkable results in the treatment of LSCD, unfortunately, due to the small size of tissue donors and the frequent need to expand cells in vitro for a long time, it is difficult to obtain sufficient cells for clinical transplantation, which results in low cell viability after transplantation. In addition, transplanted cells often develop fibrosis or degradation and their survival rates are very low. Further, problems, such as the differentiation of cultured corneal stem cells, physiological and biochemical changes, and reducing rejection after allotransplantation, need to be solved.

More importantly, the mechanism of action of transplanted stem cells in repairing damaged ocular surfaces and their fate after transplantation remains uncertain. Possible cell fates include: (1) metabolism and degradation after application, (2) remaining at the site of administration, (3) migration to damaged tissues, or (4) other unknown fates. Additionally, the regulatory process for obtaining cell therapy approval requires significant expertise, time, and investment, which may diminish the expected clinical effects of cell therapies and prolong clinical translation.

With the developments in science and technology, tissue engineering, nanotechnology, exosomes, gene therapy, and miRNAs have shown great potential. However, these cutting-edge technologies are technically demanding and costly. Most new technology experiments are conducted in animal models, lacking a comprehensive in-body evaluation of the human eye. Consequently, LSCD remain a challenge for patients, clinicians, and scientists.

Conclusions and future perspectives

This review described the anatomy, location, biomarkers, isolation and culture techniques of LESCs, as wells as the regulatory pathways of the limbal niche. Subsequently, we concluded the aetiology, clinical manifestations, diagnosis, and treatment of LSCD, especially stem cell-based

therapeutic strategies. In addition, we also innovatively summarized the latest advances and clinical translations of LESCs in recent years to provide new ideas for the treatment of LSCD.

In the future, it will be necessary to search for limbal epithelial stem cell-specific markers, as well as the characteristics and composition of limbal microenvironment. Besides, although many signaling pathways responsible for the healing stimulus and their crosstalk have been uncovered, little is known about the cell-cell, cell-ECM, and cytokine-cell interactions that regulate the self-renewal and generation of progeny cells, which is a direction for future research. What's more, the innovative in vitro culture and expansion techniques, especially 3D organoids, have broad application prospects. In addition, animal models closer to human eye diseases should be established to obtain more realistic data and achieve better clinical translations.

The ideal goal of treating LSCD is to restore the structure of the limbal niche so that new stem cells from internal or external sources can repopulate the niche and replicate successfully, enabling the corneal epithelium to regenerate and regain its transparent, uniform, and self-renewing ability. First, the consensus on the management of LSCD remains to be improved, and the use of advanced technologies, such as artificial intelligence, for early diagnosis and hierarchical treatment can be a promising method. Simultaneously, improving the availability of surgery and reducing costs and adverse reactions are the focus of clinicians' efforts and more large-scale clinical trials are needed. At the same time, how to activate the regeneration of autologous LESCs in situ, improve stem cell replication, settle in their niche, and deliver soluble factors to their environment is the focus of future research.

Recently, the local use of growth factors, novel drugs, decellularized matrices, and the introduction of new technologies, including tissue engineering, nanotechnology, exosomes, gene therapy, and miRNAs, have broadened prospects for the treatment of LSCD. In addition, RNA-seq technology can also help us further understand the function and niche regulatory properties of LESCs, including the discovery of novel, highly specific expression markers and niche regulatory components that can promote or inhibit LESC proliferation and differentiation. How to utilize new technologies and achieve laboratory-to-clinical translation for better treating LSCD is also the direction for future research.

In conclusion, integrating various technologies and a multidisciplinary approach (clinical, biological, and genetic) to personalise the treatment of patients with LSCD will be a promising strategy in the future.

Abbreviations

ADSC	Adipose-derived mesenchymal stem cells
AM	Amniotic membrane
AMT	Amniotic membrane transplantation
ATELC	Auto-tissue engineering lamellar cornea
BAC	Bioartificial corneas
bFGF	Basic fibroblast growth factor
BM-MSC	Bone marrow-derived mesenchymal stem cells
BNC	Biopolymer bacterial nanocellulose
CaMKII	Calmodulin-dependent kinase II
CLAL	Conjunctival limbal allograft
CLAU	Conjunctival limbal autograft
CLET	Cultured Llimbal epithelial transplantation
COMET	Cultivated oral mucosal epithelial transplantation
CSSC-EV	Corneal stromal stem cell-derived extracellular vesicles
DKK	Dickkopf
ECM	Extra cellular matrix
EGF	Epidermal growth factor
EV	Extracellular vesicle
Exos	Exosomes
Fzd	Frizzled
GelMA	Gelatin methacrylate
HA-DOPA	Hydrazone-crosslinked hyaluronic acid
HAGM	Hyaluronic acid glycidyl methacrylate
hASC	Human adipose-derived stem cells
hESC	Human embryonic stem cells
hESC-LSC	Human embryonic stem cell-derived limbal stem cell
hiPSC	Human-induced pluripotent stem cells
HLA	Human leukocyte antigen
hLESC	Human limbal epithelial stem cells
LESC	Limbal epithelial stem cellsLMel: limbal melanocytes
LMSC	Limbal mesenchymal cells
LSC	Limbal stem cell
LSCD	Limbal stem cell deficiency
LV	Lentiviral vector
miRNA	microRNA
MSC	Mesenchymal stem cells
MSCT	Mesenchymal stem cell transplantation
NICD	Notch intracellular domain
OEM	Onion epithelial membrane
PCP	Planar cell polarity
PLA	Polylactic acid
PCL	Polycaprolactone
pDCSM	Porcine decellularised corneal stroma matrix
PEDF	Pigment epithelium-derived factor
PUTK	Thioketal-containing polyurethane
RH	ROS -scavenging hydrogel
SC	Schwann cells
SHH	Sonic Hedgehog
SLET	Simple limbal epithelial transplantation
SSEA4	Stage-specific embryonic antigen 4
TGF	Transforming growth factor

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SL drafted the manuscript and created all the figures. SL, HS, LC and YF discussed the concepts of the manuscript. All authors read and approved the final manuscript.

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Data availability
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Declarations

Ethics approval and consent to participate
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Conflict of interest
The authors declare that they have no conflict of interest.

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