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Limbal Stem Cell Diseases

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Abstract

The function of limbal stem/progenitor cells (LSCs) is critical to maintain corneal epithelial homeostasis. Many external insults and intrinsic defects can be deleterious to LSCs and their niche microenvironment, resulting in limbal stem cell dysfunction or deficiency (LSCD). Ocular comorbidities, frequent in eyes with LSCD, can exacerbate the dysfunction of residual LSCs, and limit the survival of transplanted LSCs. Clinical presentation and disease evolution vary among different etiologies of LSCD. New ocular imaging modalities and molecular markers are now available to standardize the diagnosis criteria and stage the severity of the disease. Medical therapies may be sufficient to reverse the disease if residual LSCs are present. A stepwise approach should be followed to optimize the ocular surface, eliminate the causative factors and treat comorbid conditions, before considering surgical interventions. Furthermore, surgical options are selected depending on the severity and laterality of the disease. The standardized diagnostic criteria to stage the disease is necessary to objectively evaluate and compare the efficacy of the emerging customized therapies.

Keywords

anterior segment optical coherence tomography; *in vivo* confocal microscopy; limbal stem cell; limbal stem cell deficiency; limbus; treatment

1. Introduction

The limbus is a small and complex structure embracing the 1.5 to 2.0 mm transitional area between the cornea and sclera. It is defined anteriorly by a conceptual line drawn between Descemet and Bowman membranes, and posteriorly by a tangential line drawn from the scleral spur (Van Buskirk, 1989). The basal epithelium of the limbus contains a distinct population of adult stem cells called limbal stem/progenitor cells (LSCs) (Davanger and

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Evensen, 1971; Tseng, 1989). LSCs closely interact with their microenvironment, commonly referred to as the stem cell niche, which consists of stromal cells, melanocytes, and extracellular matrix, in a highly vascularized and innervated stroma (Dziasko and Daniels, 2016; Scadden, 2006). These interactions confer dual functions on the limbus, i.e., barrier between the corneal and conjunctival epithelium, and renewal of corneal epithelial cells (Cotsarelis et al., 1989). Both functions are necessary to maintain homeostasis of the corneal epithelium, the integrity of the ocular surface, and visual function under physiologic conditions.

Dysfunction or destruction of the LSCs and their niche, by genetic conditions, auto-immune systemic diseases, trauma or injury will result in the loss of barrier and corneal epithelial cells renewal functions, presenting as limbal stem cell deficiency (LSCD) (Tseng, 1989). The limbus is also a potential site for other degenerative and inflammatory pathologies affecting other cell types (Gueudry et al., 2019).

LSCD has been recognized as a distinct clinical disease for more than 2 decades (Tseng, 1989). Clinically, the loss of limbal function is characterized by cellular invasion onto the cornea by conjunctival epithelium, leading to impaired epithelial wound healing. This results in chronic ocular surface inflammation, neovascularization, and eventually opacification. Recurrent epithelial erosions, corneal ulcers, or even corneal perforation can also occur as complications of LSCD (Le et al., 2018c). Reduction in vision can be severe to the legal blindness level (Flaxman et al., 2017). To overcome the lack of agreement on the diagnostic criteria, staging, classification, and management of LSCD, global consensuses on LSCD developed by the International LSCD Working group have recently been published, which aim to provide a better understanding of the disease, and general guidelines to classify, diagnose, stage and manage the disease (Deng et al., 2019; Deng et al., 2020b).

Strategies for restoration of the ocular surface, via repopulation of LSCSs and decrease inflammation, improvement of ECM, restoration of innervation, providing soluble growth factors in the niche environment, have been reported to be successful to restore LSC function in eyes with partial LSCD (Cheung et al., 2020; Holland, 1996; Holland and Schwartz, 2004; Le et al., 2020b), but because there has not been an agreement on the disease entity until recently, comparisons of the efficacy among different strategies remain impossible (Le et al., 2020b). For the same reason, estimation of the prevalence of LSCD is largely unknown. This review provides an overview of the pathologic conditions that affect the limbus, and summarizes the biology of LSCs, pathophysiology of diseases involving the limbus, recent advances in LSCD diagnostic, staging, treatment strategies, and emerging therapies.

2. Evidence of Limbal Stem Cells Location

The most accepted hypothesis for repair and regeneration of human corneal epithelium is based on the X-Y-Z hypothesis initially described by Thoft and Friend (Thoft and Friend, 1983). In this hypothesis, X is the anterior migration of cells from the basal limbal epithelium, Y is the centripetal migration of peripheral cells from the limbus, and Z is the loss of corneal epithelial cells from the surface. The epithelial cell number in the cornea can

be determined using the equation Z = X + Y. This equation describes the limbus as the source of cells for corneal epithelial cell renewal. Transdifferentiation of conjunctival cells into corneal epithelial cells has first been proposed as a mechanism of corneal epithelial healing (Shapiro et al., 1981). This hypothesis was later refuted, in favor of the X-Y-Z hypothesis. Indeed, long term follow-up studies in animal models of partial and total limbal removal have confirmed that a conjunctival epithelium phenotype move along the limbal margin to cover the corneal surface (Chen and Tseng, 1991). In less severe corneal wounds, the epithelium covering the affected region may temporarily derived from the conjunctiva, until the remaining functional limbal-derived progenitors are mobilized to replenish the damaged corneal epithelium (Moyer et al., 1996). This concept is reinforced by the fact that epithelial proliferation and wound-healing is impaired after partial to total limbal ablation (Huang and Tseng, 1991). Moreover, transplantation of limbal tissue, or limbal derived cell grafts, can successfully restore severely damaged ocular surfaces (Kenyon and Tseng, 1989; Le et al., 2020b; Pellegrini et al., 1997).

LSCs express a set of molecular markers of stem and progenitor cells in other tissues (Pellegrini et al., 2001; Schlotzer-Schrehardt and Kruse, 2005). LSCs have stem cells characteristics, including the capacity of indefinite cell renewal while staying undifferentiated, whereas progenitors are early descendant of stem cells, considered the initial step of the tissue differentiation. They divide more frequently than stem cells but have a limited proliferative potential (Potten and Loeffler, 1990). LSCs also have a long life-span and are quiescent state under homeostatic conditions, due to their slow cycling activity. LSCs exhibit a high capacity for self-renewal and proliferation (Dua and Azuara-Blanco, 2000). However, technically LSCs cannot be distinguished from their immediate progenitor cells because of a lack of specific biomarkers. During normal homeostasis, corneal epithelial renewal is coordinated mostly by their progenitors' division, differentiation and migration. During corneal wound-healing or disease, the dynamics of LSCs and their progenitors' division is amplified to cope with the increased cell loss (Chen and Tseng, 1991; Huang and Tseng, 1991).

More recently, a controversial hypothesis mainly based on animal models has suggested that the stem cells are located in the basal layer throughout the cornea, and that LSCs are not necessary to maintain corneal homeostasis (Majo et al., 2008). Further, in pathologic eyes in human, when the limbus is not visible, LSCs may relocate under a pannus or a corneal neovascularization (Dua et al., 2009; Le et al., 2018b). This corneal hypothesis led to the development of corneo-limbal epithelial cells lineage tracing animal models, using genetic fluorescent markers that allow for the monitoring of cell fate and progeny (Amitai-Lange et al., 2015; Di Girolamo, 2015). Nasser et al. showed that following complete destruction of all limbal epithelial cells, but if the limbal niche remains intact, the limbal epithelium might be restored by the dedifferentiation of committed corneal epithelial cells in mice (Nasser et al., 2018). Given the high number of cell divisions required to replenish the LSC pool, it is unlikely that a small population of central corneal stem cells are responsible for LSC recovery in mice (Blanpain and Fuchs, 2014; Nasser et al., 2018). Dedifferentiation of committed corneal epithelial cells if indeed occurs, could provide a potential approach to treat LSCD, alleviating the need for allogenic LSCs transplants. However, to date, such committed corneal epithelial cells have only been described in animal models and remain to

be identified in humans. This study further emphasizes the critical role of a healthy LSC niche in the maintenance of LSCs. In contrast, other studies using animal models showed that during normal homeostasis, corneal epithelial cells move centripetally from the limbus, as predicted by the X-Y-Z hypothesis (Di Girolamo, 2015; Dorà et al., 2015; Seyed-Safi and Daniels, 2020). Corneal progenitor cells could be found in the central cornea in mice while they are absent in human. Therefore, the observation from mice does not necessarily translate to LSC regulation and corneal wound repair in human.

3. The role of limbal niche

Niches are three-dimensional, stem cell-sheltering, highly organized microenvironments that is usually localized at tissue transition zones (Mcnairn and Guasch, 2011). Stem cells rely on interactions with their immediate niche to proliferate, migrate and differentiate (Davanger and Evensen, 1971; Lane et al., 2014). Regulation of proliferation and differentiation of LSCs are the result of complex molecular cross-talk with neighboring niche cells, soluble factors, and extracellular membrane (ECM) components (Scadden, 2006, 2014).

In the human eye, the palisades of Vogt (POV) (Goldberg and Bron, 1982), limbal crypts and focal stromal projections (Dua et al., 2005; Shortt et al., 2007), and limbal lacunae (Zarei-Ghanavati et al., 2011) are different structures of the limbal niche (Seyed-Safi and Daniels, 2020). Compared with other stem cell niches, a unique advantage of the limbal niche is its direct visualization by non-invasive *in vivo* imaging including slit lamp biomicroscopy, anterior segment optical coherence tomography and *in vivo* scanning confocal microscopy, IVCM. Characterization of the precise architecture and molecular composition of the limbal niche, yet important to improve LSC culture models (Gonzalez et al., 2016; Mei et al., 2014).

Various niche cell types have been described, including immune cells (Vantrappen et al., 1985), limbal stromal cells (Dziasko et al., 2014), melanocytes (Davanger and Evensen, 1971; Dziasko et al., 2015; Hayashi et al., 2007; Higa et al., 2005), corneal mesenchymal cells (Kureshi et al., 2015; Nakatsu et al., 2014), nerves, and vascular cells (Notara et al., 2018). The mounting evidence suggests that LSCs are supported by accumulative effects of different niche cell types. A wide range of specific ECM components have been identified in the limbus, such as the lack of collagen XII (Wessel et al., 1997), the presence of a specific collagen IV, laminin isoforms (Ding et al., 2008; Ljubimov et al., 1995), and tenascin C (Schlotzer-Schrehardt et al., 2007). Heavy chain hyaluronan (HA) /pentraxin 3 is one of the components that might be responsible for the therapeutic benefit of amniotic membrane in ocular surface reconstruction (Chen et al., 2015; Tseng, 2016). Recent finding from HA knockout experiments in mice suggests a role of HA in the maintenance of LSCs and corneal epithelial wound healing (Gesteira et al., 2017). Besides maintaining the architecture of the limbal niche, the dynamic nature of ECM plays a major role in the molecular regulation of stem cells by providing external cue and controlling the level of growth factors in the niche (Seyed-Safi and Daniels, 2020).

Interactions between LSCs, specialized ECM components, signaling molecules, and niche cells regulate the behavior of the LSCs, and their ability to promote corneal epithelial

maturation and renewal (Dziasko and Daniels, 2016; Mei et al., 2012; Nakatsu et al., 2014; Seyed-Safi and Daniels, 2020). Animal models also showed that limbal stroma could maintain the stemness of LSCs, while corneal stroma could promote cell differentiation, proliferation and apoptosis (Espana et al., 2003). It is important to note that injuries or disturbance involving any components of the limbal niche can result in LSC dysfunction and failure. Disruption of the normal corneal epithelial differentiation and maturation without LSC depletion can manifest as LSCD. Distinguishing the underlying pathophysiology of corneal epithelial failure is necessary to reach a rational treatment approach.

4. Etiologies of limbal stem cell diseases

Both genetic defects and acquired conditions can lead to LSC diseases (Table 1). Descriptive studies focusing on demographic influence and causes for LSCD are limited (Vazirani et al., 2018), since most studies focus on describing treatment outcomes. Vazirani and colleagues recently provided a comprehensive description of LSCD demographics and causes over a 10-year study period, including nearly 2000 eyes from 2 tertiary centers in Asia (Vazirani et al., 2018). The majority of patients presented total unilateral LSCD and were children or young males, with a vision of 20/200 or less in the affected eye. In cases of unilateral LSCD, the most frequent causes were ocular burn (84%) followed by Stevens-Johnson syndrome (SJS; 4%), atopic keratoconjunctivitis (3%), mucous membrane pemphigoid (MMP), and traumatism (2% each). In cases of bilateral LSCD, the major causes were atopic keratoconjunctivitis and ocular burn (30% each) followed by SJS (23%), aniridia (9%), and MMP (4%). Because the study was hospital-based, the prevalence of the disease is likely underestimated. Less severe disease, such as mild to moderate LSCD may not have been included. In addition, the study was performed in Asia, and the prevalence of each cause may be different in Western countries where chemical injuries are far less common. Contact lens wear and iatrogenic LSCD have been found to be the leading causes of LSCD in Southern California (Deng et al., 2012).

Among LSC diseases, mechanisms of LSC damage, clinical presentation, degree of LSC dysfunction, laterality and clinical evolution can vary and progress over time. Any additional comorbidity, such as ocular surface inflammation, preservatives or systemic chemotherapy, eyelid abnormalities, and neurotrophic keratopathy can compromise or exacerbate the dysfunction of residual LSCs. Understanding the different mechanisms leading to LSC diseases and LSCD is critical to target appropriate treatments for specific causes. For example, in prolonged contact lens-wear, LSC dysfunction can occur due to a combination of hypoxic, mechanical, and toxic insults, that are most frequently localized on the superior limbus (Martin, 2007). If residual LSCs are present, LSCD can be asymptomatic, and is likely reversible upon intensive medical management, including terminating contact lens-wear (Jeng et al., 2011; Kim et al., 2014). Conversely, when LSCs or their niche are severely damaged, clinical presentation of LSCD involves the entire corneal surface, and medical management is insufficient, thus requiring surgical intervention (Shen et al., 2015). LSC dysfunction associated with glaucoma surgery also exhibits specific clinical features, as the site of glaucoma surgery is strongly correlated with the clinical location of LSC dysfunction and LSCD (Sun et al., 2020). In the event of chemical or thermal burn, the degree of niche and LSC destruction depends on the severity of the insult, where the most severe leads to

total LSCD (Basu et al., 2016). Other ocular damages are frequent, thus loss of corneal transparency and conjunctivalization occur quickly and are often permanent, requiring surgical management.

LSC dysfunction could result from chronic inflammation with concomitant immune-mediated changes, such as in SJS, MMP, vernal, and atopic conjunctivitis. If the inflammation is not aggressively treated by intensive therapies, LSC dysfunction could progress to corneal opacification over time in MMP, and shield ulcer in vernal/atopic conjunctivitis (Williams et al., 2011). In MMP, chronic inflammation combined with poor tear film constitutes a chronic negative environment for the LSCs (Schmidt et al., 2008). Initial clinical signs may not be specific and often very subtle, including chronic conjunctivitis and tear dysfunction (Mondino and Brown, 1981). Morphologic changes of the epithelial cells are apparent in the central cornea prior to conjunctivalization, leading to a difficult clinical evaluation if ancillary testing is not performed (Vera et al., 2009).

In congenital LSC diseases, phenotypic spectrum and variable expressivity of the conditions make clinical diagnosis and classification difficult. In congenital aniridia, replacement of the corneal epithelium with a conjunctival phenotype occurs over a time span of years to decades. This is a slow degenerative process termed aniridia-associated keratopathy (AAK) (Ihnatko et al., 2016). Functioning LSCs can be found at early stages of AAK (Edén et al., 2010). Corneal epithelial phenotypic changes are correlated with the type of PAX6 mutations identified, and worsen with aging (Vasilyeva et al., 2020). Early phenotypic changes can be seen on the central cornea by IVCM, even when the conjunctival invasion is limited to the limbal area and the central cornea is unaffected (Lagali et al., 2018). In addition, in this condition, conjunctiva is abnormally maintained in a proangiogenic and proliferative state, depending on the PAX6 mutation (Latta et al., 2020). Similarly, in ectodermal ectrodactyly syndrome, the level of LSCs dysfunction also worsened with aging, but has not been found to correlate with an underlying molecular defect in the p63 gene (Di Iorio et al., 2012).

Other ocular surface diseases can affect the function of corneal epithelial cells. For example, in vitamin A deficiency, cell differentiation and apoptosis are altered (Barber et al., 2014). In severe dry eye disease, chronic inflammation can lead to morphologic changes and keratinization (Matsumoto and Ibrahim, 2018). In ocular surface squamous neoplasia (OSSN), tumorous cells overtake the normal function of LSCs and invade onto the cornea (Kieval et al., 2012). Once the tumor is medically treated, LSC function returns to normal. In extensive OSSN that affects more than 50% of limbus, surgical excision with cryotherapy could cause iatrogenic LSCD (Deng et al., 2019). Pterygium is considered a defect in the barrier function of limbus where conjunctival fibrosis invades onto the cornea. Once the barrier function is restored, LSCD does not generally occur, as there is a sufficient amount of functional LSCs (Kaufman et al., 2013).

Based on the different mechanisms of LSC diseases, animal models, such as sulfur mustards gas injury models (Kadar et al., 2009; Ruff et al., 2013), topical administration of high concentrations of benzalkonium chloride models (Lin et al., 2013), and transgenic PAX6 mouse models (Li et al., 2015) have been established. Information obtained from these

models will help in the development of appropriate treatments for LSCD arising from different causes.

In summary, the amount of remaining LSCs and their function seem to play a role in the various degrees of LSCD, clinical presentation, and clinical course (Shimazaki et al., 2007). It has been proposed that approximatively 25–33% of the limbus must be intact to ensure normal ocular resurfacing (Tseng, 1989). However, functional LSCs can also be found in eyes with clinical signs of total LSCD (Le et al., 2018b). Objective evaluation of LSC function, by clinical examination combined with additional diagnostic imaging and/or molecular modalities, is critical to fully capture their level of function.

5. LSCD presentation, diagnosis and grading

a. Clinical presentation

The diagnosis of LSCD consists of medical history, clinical presentation, and diagnostic tests (Deng et al., 2019; Le et al., 2018c). Most frequent symptoms include decreased vision, recurrent episodes of pain, chronic conjunctival hyperemia, foreign body sensation, tearing, blepharospasm and/or ocular discomfort. These symptoms are related to poor epithelial wound healing. In addition, chronic non-healing epithelial defect is a common manifestation of severe LSCD, increasing the risk of microbial infection (Sandali et al., 2012).

Compared with normal eyes, early signs of LSCD include irregular corneal epithelium and alteration or loss of POV (Lagali et al., 2013; Le et al., 2017). The irregular epithelium varies in thickness and transparency and can be constituted of either a mixture of metaplastic corneal epithelial cells and conjunctival cells, or only conjunctival cells. Fluorescein stains the basement membrane. Unlike corneal epithelial cells, abnormal and conjunctival epithelial cells lack tight junctions. The fluorescein staining pattern is characterized by a stippling or granular staining of the area covered by the conjunctival epithelium (Le et al., 2018c). For this reason, the late abnormal fluorescein staining remains after several minutes. Fluorescein tends to pool on the affected area because the thinner abnormal epithelium (Dua, 1998). A clear line of demarcation may be visible in sectoral LSCD, between the area covered by the corneal and conjunctival epithelial cells (Figure 1A–C).

As the disease severity increases, the conjunctival epithelium becomes confluent and tends to spread in a spiral pattern from the limbus onto the cornea, thereby invading the visual axis and leading to a vortex pattern keratopathy which can be visualized by fluorescein. Recurrent epithelial erosions, mild anterior stromal haze and superficial vascularization are also usually seen. Furthermore, severe to total LSCD results in almost complete absence of normal corneal epithelium and subsequent diffuse corneal stromal haze. Stromal scarring, and corneal opacification also often occur, leading to functional blindness (Figure 1D). Clinical presentation of chemical burns can exhibit a thick pannus and corneal neovascularization in the affected area. However, neovascularization in the deep stroma level is common but not pathognomonic to severe LSCD (Le et al., 2018c).

b. Grading system of clinical presentation

Detection and interpretation of the clinical signs of LSCD are subtle in mild stages and remain subjective, since many of them can be found in other corneal diseases. A recent international consensus established an objective grading that could be adapted readily among all ophthalmologists (Deng et al., 2019). LSCD is categorized into 3 stages (I to III) based on the extent of corneal and limbal involvement detected by clinical examination (Table 2). For a more precise evaluation and monitoring of the disease severity, another grading system was developed (Aravena et al., 2019), based on the limbal involvement in clock hours (1–4 points), the corneal surface area affected (1–4 points), and the visual axis involvement (0 or 2 points). LSCD is then classified as mild (1–4 points), moderate (5–7 points) or severe (8–10 points) (Figure 2). The grades are correlated with the decrease in basal cell density (BCD) observed in the central cornea, the visual outcomes, and the stages I to III defined by the global consensus. The global consensus also recommends that additional diagnostic tests, molecular or by anterior segment imaging, should be performed in addition of the clinical examination, if possible (Deng et al., 2019).

c. Additional diagnostic methods

i. Impression cytology—Impression cytology is a well-established method to diagnose LSCD, considered as a gold-standard. The presence of goblet cells on the cornea indicates its invasion by conjunctival cells, defining LSCD (Puangsricharern and Tseng, 1995). The sensitivity of impression cytology is affected by many factors that have a direct effect on the number of cells harvested, such as the filter pore size, the surfactant treatment used, and the pressure applied to the membrane (Singh et al., 2005). Finally, the location of the sample is important, especially in sectoral LSCD. Immunohistochemical analysis of impression cytology specimens is now performed to identify the specific type of cytokeratins expressed and determine the type of epithelium. Noteworthy, is the lack of exclusivity of cytokeratin (K) expression in ocular tissues. K12 is marker of differentiated corneal epithelial cells, whereas K13 and K7 are conjunctival-epithelial cell markers (Poli et al., 2015; Ramirez-Miranda et al., 2011).

ii. In vivo laser scanning confocal microscopy—The use of IVCM in the diagnosis of LSCD has expanded exponentially over the last decade, mostly using the Heidelberg Retina Tomograph with Rostock Cornea module (Heidelberg Engineering, GmbH, Heidelberg, Germany), that can obtain images of the limbus of better quality compared to the other available devices (Tavakoli et al., 2008). IVCM is useful in observing the significant microstructural changes that occur in the limbal area. Initially, the basal epithelial cells borders become less distinct, while the nuclei become prominent; they later become larger and metaplastic (Deng et al., 2012). In addition, the basal epithelial cell and subbasal nerve densities, and the epithelial thickness decrease dramatically (Chan et al., 2015a; Chan et al., 2015b). Other subbasal nerve morphologic changes occur, including increased tortuosity (Caro-Magdaleno et al., 2019; Chuephanich et al., 2017). These criteria, measured in the central cornea, are correlated with the clinical grading of the disease. They can serve as *in vivo* evaluation tools of LSC function (Aravena et al., 2019; Chuephanich et al., 2017; Le et al., 2017).

iii. Anterior segment optical coherence tomography—Anterior segment optical coherence tomography (AS-OCT) can image ocular anterior segment structures, including the cornea and the limbus with a spectral-domain resolution of 3 μm (Feng and Simpson, 2005). Consistently with IVCM findings, the central and limbal epithelial thicknesses decreased in eyes with LSCD and correlated with the LSCD severity (Liang et al., 2020). This imaging modality can help to guide diagnosis when LSCD is suspected.

The epithelial thickness can be measured automatically within 6 or 9 central millimeters (Yang et al., 2014). The automated measurements have been reported in normal eyes and in many ocular disorders (Francoz et al., 2011). In LSCD, manual measurements should be preferred because changes occurring in the epithelium and in the underlying stroma in case of scarring can interfere with the automated epithelial thickness measurements (Le et al., 2018a). We reported that limbal structures can be visualized using AS-OCT and varies among ethnicities (Le et al., 2018a). However, IVCM remains a necessary test to precisely evaluate LSC function.

Optical coherence tomography-angiography (OCT-A) allows visualization of corneal and limbal vasculature (Ang et al., 2015). However, as corneal neovascularization is not a specific sign of LSCD, OCT-A cannot be used to quantify the severity of LSCD. The development of new three-dimensional imaging modules acquisition, reconstruction, and analysis techniques are being investigated and should provide structural information on the LSC niche in both normal and pathologic conditions (Bizheva et al., 2011; Grieve et al., 2015).

In summary, each of these changes, epithelial thinning, reduction of subbasal nerve density and corneal BCD is not specific of LSCD and can be found under other systemic diseases, ocular disorders, therapies or surgeries or simply due to aging (Zheng et al., 2016). However, the large-scale reduction of BCD and subbasal nerve plexus density have only been reported for LSCD (Chan et al., 2015a; Chuephanich et al., 2017), which has been confirmed by others (Caro-Magdaleno et al., 2019). The combination of all these microstructural changes is mainly seen in LSCD and could be considered the characteristic signs of LSCD. It can be still challenging to distinguish LSC dysfunction from lack of LSCs in some situations.

Clinical experience in LSCD is necessary to provide an accurate diagnosis, including staging of the LSCD severity, acquisition, and analysis of IVCM images. While AS-OCT is widely available to measure central epithelial thickness when the disease is suspected, IVCM and a laboratory to process the impression cytology are usually available only in specialized tertiary centers and require trained examiners. Patients referral to an eye care center where is equipped with necessary facility and expertise in LSCD should be considered when the disease is suspected to confirm, and stage the disease using appropriate imaging and laboratory diagnostic tests.”

6. Management

The first global consensus in the medical and surgical management of LSCD has been recently published (Deng et al., 2020b). Accurate diagnosis of LSCD is the first step in

managing patients presenting with LSCD. In all cases, time and effort should be taken first to optimize the ocular surface by controlling causative factors and comorbid conditions. This includes treatment of autoimmune diseases and chronic ocular inflammation by immunosuppression and/or topical steroids, and the eradication of an infection or iatrogenic insult. Comorbid conditions such as dry eye disease and exposure keratopathy are amenable to various conservative options such as lubrication, punctal occlusion, anti-inflammatory eye medications, and autologous serum tears. If medical treatments are not sufficient, large diameter scleral lenses could be tried before considering surgical treatment. If necessary, fornices and eyelid reconstruction would be the next step to remove the conjunctival cicatricial tissues and correct the abnormality of eyelids or trichiasis.

Once the ocular surface is optimized, additional therapeutic options range from conservative to invasive and depend on the cause, severity and laterality of the disease. The approach to therapies follows a stepwise flowchart that is detailed in the global consensus (Figure 3) (Deng et al., 2020b). Briefly, stages I and IIA are managed medically and superficial keratectomy with amniotic membrane can be considered in progressive cases. Further surgical strategies are reserved for stages IIB and III, including various types of direct or *ex vivo,* autologous or allogenic, LSCs transplants (Borderie et al., 2019; Cheung et al., 2020; Rama et al., 2010; Sangwan et al., 2012). Le et al. reported that autologous transplants seem to have higher retention and lower complications rates compared to allogeneic grafts, and are preferred when treating unilateral LSCD (Le et al., 2020b). The meta-analysis showed that direct autologous LSC transplantation and cultivated autologous transplant may have similar outcomes. Improvement of the ocular surface occurred in 85.7% (95%CI, 79.5%–90.3%) of eyes after direct autologous LSC transplantation and in 84.7% (95%CI, 77.2%–90.0%) after cultivated autologous LSC transplantation. However, randomized clinical trials using objective criteria to stage the disease are still necessary to confirm these findings (Le et al., 2020a).

In bilateral cases, allogeneic LSC transplantations can be considered, but require lifetime oral immunosuppression (Cheung et al., 2020; Eslani et al., 2019; Movahedan et al., 2017). Ocular surface improvement appears to be lower in allogeneic transplants, for both direct (57.8%, 95%CI, 49.0%–66.1%) and cultivated (63.2%, 95%CI, 49.3%–75.2) LSC transplantation than in autologous transplants (Le et al., 2020b). Elective patients can be treated with cultivated oral mucosal epithelial transplantation, however randomized trials evaluating its efficacy compared with allogeneic LSC transplantation are lacking. Finally, keratoprostheses have been used to treat bilateral severe LSCD. Fast visual rehabilitation makes this treatment advantageous without the requirement of oral immunosuppression. Yet, despite early success of visual recovery, the device retention and sight threatening complications rates worsen with the follow-up (Aravena et al., 2016).

7. Limbal stem cell expansion and emerging therapies

In vitro cultivation of human epithelial cells was developed by Howard Green in the 60's (Todaro and Green, 1963). Initially, this model of stem cell expansion consisted of a co-culture of human epidermal keratinocytes with 3T3-mouse embryonic fibroblasts, functioning as feeder cells. It led to the first stem cell therapy using cultured cells (Green et

al., 1979; O'Connor et al., 1981; Rheinwald and Green, 1975a, b). It was later applied to LSCs (De Luca et al., 2006). Pellegrini et al. first reported that autologous transplants of cultivated LSCs on irradiated 3T3 fibroblasts, obtained from a 1–2 mm^2 limbal biopsy, restored the corneal surface in 2 patients with total LSCD from chemical burn (Pellegrini et al., 1997). They later confirmed that this approach could maintain the ocular surface over the time, providing good long-term visual outcomes (Rama et al., 2010).

Different biological and synthetic materials that mimic the LSCs niche can be used as carrier for LSCs culture, such as amniotic membrane, fibrin, and silicon hydrogel contact lenses (Hynds et al., 2018; Nguyen et al., 2018). Three dimensional LSCs niche models are also being studied (Dziasko et al., 2015; Mei et al., 2014; Nakatsu et al., 2014). Emerging therapies include small molecules promoting LSCs proliferation (González et al., 2019; Zhang et al., 2020) and extracellular vesicles derived from corneal stromal stem cells, that could inhibit inflammation and promote corneal re-epithelization (Deng et al., 2020a).

8. Conclusion

There are many causes that can alter LSC function and lead to LSCD. The clinical presentation of the resulting LSC diseases vary and is therefore not sufficient to evaluate the level of function of the remaining LSCs. New non-invasive imaging tests such as IVCM and AS-OCT are available and should be performed to confirm the diagnosis and objectively stage LSCD. The same criteria to stage LSCD should be used internationally to assess the clinical outcomes of different therapies. To date, better and more convenient treatment options for LSCD are emerging, such as *ex vivo* LSC culture and autograft. These evolving techniques in LSC transplantation should all follow good manufacturing practices guidelines. In addition, while detailed interaction and signaling pathways between LSCs and niche micro-environment are not fully understood, research shows promise in the development of new pharmacological options that could stimulate remaining dormant LSCs of the affected eye (*in vivo*) or LSC *ex vivo* cultures.

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Highlights

- Regeneration of the corneal epithelium involves self-renewal, migration and differentiation of limbal stem cells.
- Both intrinsic and extrinsic causes can destroy limbal stem cells and their microenvironment, resulting in limbal stem cell deficiency.
- Clinical presentation, evolution and prognosis can vary among etiologies of limbal stem cell deficiency.
- Diagnosis and staging of limbal stem cell deficiency requires standardized criteria.
- Optimization of the ocular surface remains the first step in managing limbal stem cell deficiency.

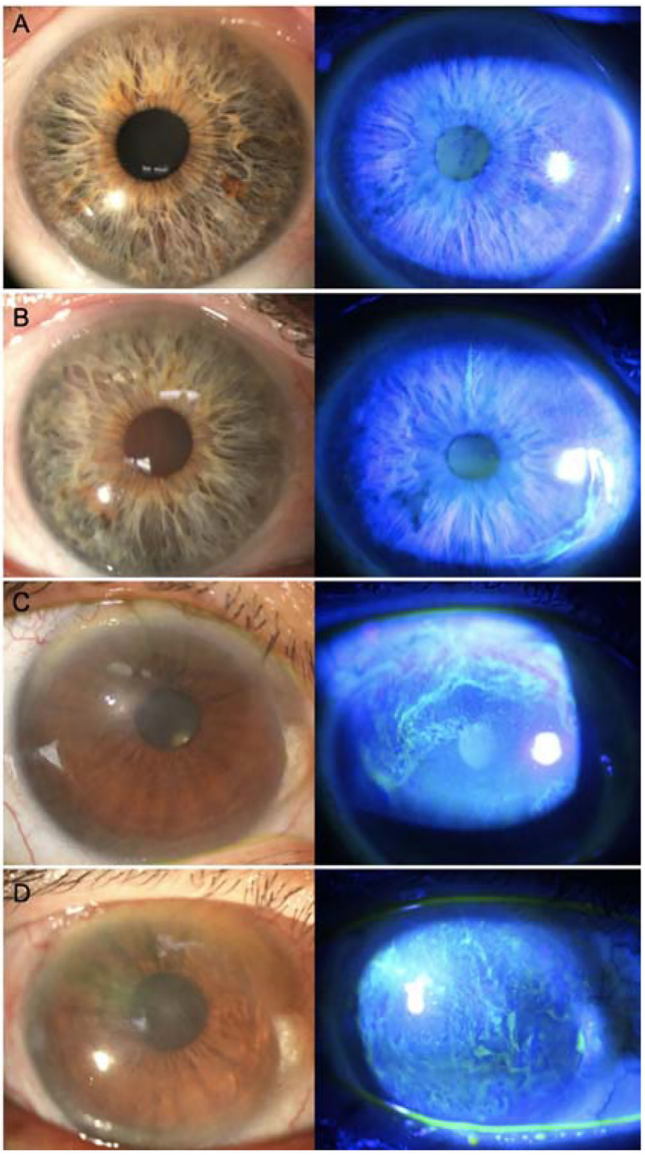

Figure 1.

Slit lamp examination in eyes presenting with LSCD

Figure 1A: Normal eye under bright light (left panel) and blue cobalt filter (right panel).

Figure 1B : Mild stage of LSCD: unremarkable examination under bright light (left panel). Superior linear granular fluorescein staining (right panel).

Figure 1C: Moderate stage of LSCD: sectoral irregular epithelial reflex with opacification under bright light (left panel). Fluorescein staining showing a demarcation line between the clear and affected areas, involving the visual axis (right panel).

Figure 1D: Severe stage of LSCD: diffuse epithelial opacification and corneal haze under bright light (left panel). Diffuse vortex pattern fluorescein staining (right panel).

LSCD = limbal stem cell deficiency

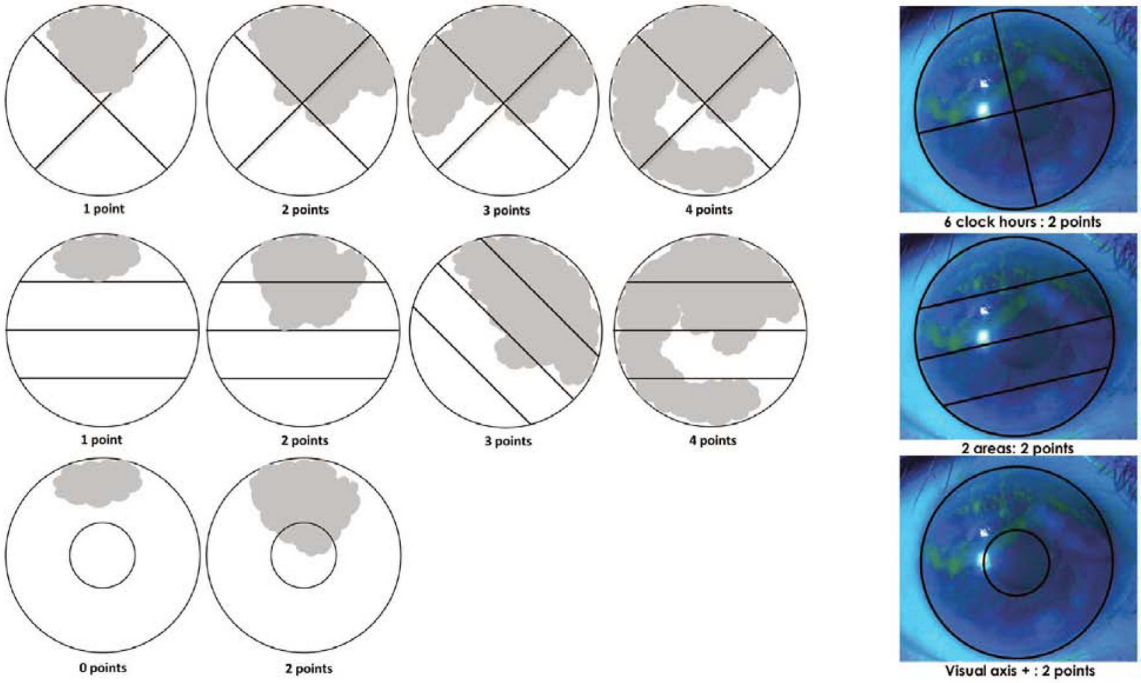

Figure 2.

Clinical grading system

The grading system includes the evaluation of the limbal involvement in clock hours (top panel), of the corneal surface involvment in areas (central panel) and of the visual axis (bottom panel). Mild score corresponds to 1–4 points, moderate to 5–7 points, and severe to 8–10 points. The eye represented exhibits a total score of 6 points (moderate stage). Reprint from Aravena &al., Cornea 2019.

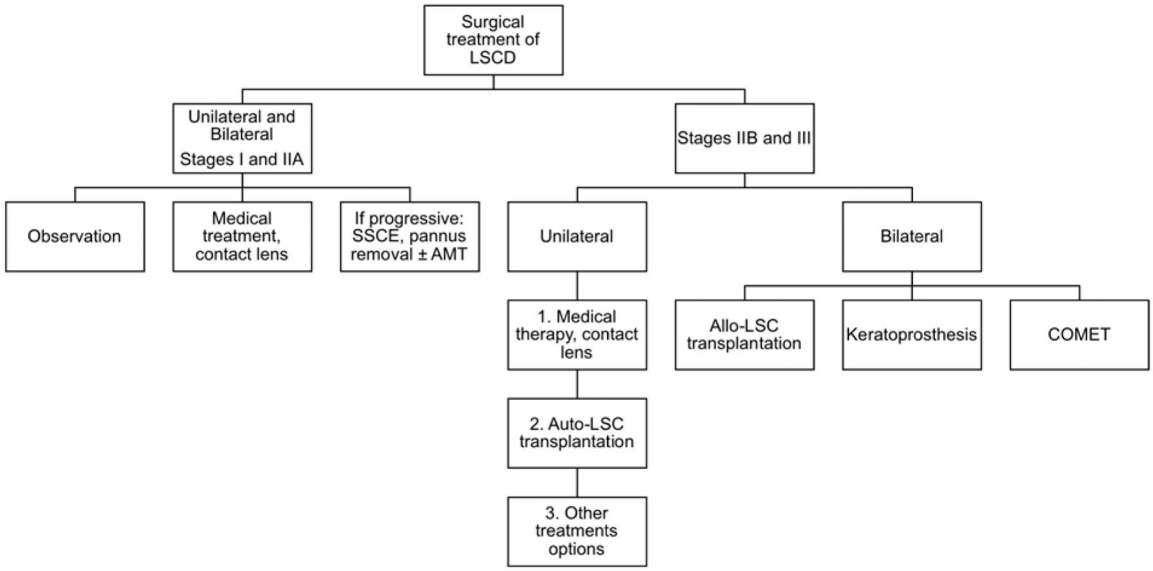

Figure 3.

Management of LSCD

Autologous LSCs transplantation includes KLAU, CLAL, autologous SLET, ex vivo-cultivated LSC autograft. Allograft include KLAL, CLAL, ex vivo cultivated LSC allograft. AMT = amniotic membrane; CLAL = conjunctival limbal allograft; COMET = cultivated oral mucosal epithelial transplantation; KLAL = keratolimbal allograft; KLAU = keratolimbal allograft; SLET = simple limbal epithelial transplantation; SSCE = sequential sectorial conjunctival epitheliectomy.

Reprint form Deng &al., Cornea 2020.

Table 1.

Major etiologies of limbal stem cell deficiency

Causes	Comments	References
Acquired		
Non-immune related		
1. Trauma	Leading cause (60–80%). Young male and children > female, unilateral > bilateral. At acute stage > 50% of limbal ischemia is a risk factor for LSCD.	
Chemical burn	Alkaline burns are more severe than acid burns (saponification of cells membrane penetrate deeper in the tissue). Sulfur mustard (chemical warfare) induces delayed LSCD.	(Haring et al., 2016; Kadar et al., 2009; Puangsricharern and Tseng, 1995)
Thermal burn	Less frequent than chemical burns.	(Puangsricharern and Tseng, 1995)
2. Iatrogenic		
Contact lens wear	Frequent cause of LSCD. Mostly reversible after CL wear discontinuation and medical treatment. Due to poor contact lens fitting, low oxygen permeability of the contact lens material, prolonged wear, sensitivity to the toxicity of CL cleaning and storage solutions.	(Donisi et al., 2003; Puangsricharern and Tseng, 1995)
Ocular surgery involving the limbus	Often leading to sectoral LSCD by direct destruction of the LSCs and the limbal niche. Excision of conjunctival and limbal tumors, extensive pterygium excision, trabeculectomy with the use of anti-metabolites are the most frequent surgeries in cause.	(Holland and Schwartz, 1997; Puangsricharern and Tseng, 1995; Sridhar et al., 2001)
Radiation	Rare, < 5% of all causes.	(Fujishima et al., 1996)
Bullous keratopathy	In case of long-standing advanced bullous keratopathy. Associated with squamous metaplasia of corneal epithelium. Usually improved after corneal transplantation.	(Paris Fdos et al., 2010; Uchino et al., 2006)
3. Toxicity from medications		
Mitomycin-C	Intraoperative and topical use.	(Dudney and Malecha, 2004)
5-Fluorouracil		(Pires et al., 2000)
Preservatives		(Lin et al., 2013)
Systemic chemotherapy	Hydroxy-urea, hydroxycarbamide.	(Ellies et al., 2001; Kim and Kim, 2015)
4. Severe Infection	Necrosis of LSCs due to severe inflammation of the ocular surface and to the toxicity of anti-microbial medications. After the acute stage of infection: progressive fibrosis of the ocular surface and limbal area. In case of trachoma: eyelid abnormalities cause chronic microtrauma of the corneal surface.	(Donisi et al., 2003; Puangsricharern and Tseng, 1995)
5. Severe blepharitis-rosacea	Chronic and is often associated with other severe ocular surfaces diseases, <5% of all causes.	(Dua and Azuara-Blanco, 1999)
Immune related		
Mucous membrane pemphigoid	Clinically: chronic relapsing inflammation and subconjunctival fibrosis, fornices shortening and obliteration, symblepharon and ankyloblepharon. Severe dry eye (from lacrymal duct scarring) may exacerbates LSCs dysfunction. The alterations of the adnexa and the microtrauma blink-related exacerbate inflammation of the ocular surface, weakening stromal support in the limbus, and leading to the gradual loss of LSCs.	(Eschle-Meniconi et al., 2005; Mondino and Brown, 1981)

Causes	Comments	References
Severe atopic and vernal keratoconjunctivitis	Due to chronic and severe ocular inflammation, mechanical trauma and palpebral scarring. Can be circumferential in severe cases. Eosinophils and inflammatory cells can cause direct destruction of LSCs.	(Daya and Ilari, 2001; Solomon et al., 2002; Samson et al., 2002; Sangwan et al., 2011)
Stevens-Johnson syndrome / Toxic epidermal necrolysis	Rare hypersensitivity reaction of the skin and the mucous membranes that can involve the ocular surface, leading to the destruction of the LSCs. Primary corneal involvement is not common, LSCD more results from the intense inflammation during the acute phase followed by secondary corneal involvement because of chronic inflammation and microtrauma from structural anomalies of eyelid position, trichiasis or symblepharon. Scarring of lacrymal gland duct orifices and the destruction of meibomian glands orifices may lead to a combination of severe dry eyes exacerbating LSCD.	(Ma et al., 2016; Puangsricharern and Tseng, 1995; Tseng, 1989; Tseng and Tsubota, 1997)
Graft-versus-host disease	Common after allogeneic hematopoietic stem cell transplantation. Fibrosis in the limbal area is stimulated by chronic inflammation. Associated dry eye further exacerbates the chronic inflammation, both detrimental to LSCs.	(Dietrich-Ntoukas et al., 2012; Meller et al., 2009)
Congenital and hereditary		
Aniridia	Prevalence: 1/50,000 to 1/100,000 newborns. Autosomic dominant or sporadic mutations of the *PAX6* gene. Aniridia can be associated with other ocular manifestations: glaucoma, cataract, foveal and optic nerve hypoplasia. The cornea, the ocular surface and the LSCs function are normal at birth, and the LSCD (called in these cases aniridia associated keratopathy, AAK) develops in the teenage years and progresses to total LSCD with stromal opacities.	(Dudakova et al., 2018; Lee and Colby, 2013)
Ectrodactyly-ectodermal dysplasia-clefting (EEC) syndrome	Heterozygous mutations of the *p63* gene. Progressive LSCD leading to visual impairment. No relationship of LSCD severity with the clinical and genetic severity of EEC syndrome.	(Di Iorio et al., 2012; Tijmes et al., 1997)
Kidney-ichthyosis-deafness (KID) syndrome	Rare congenital ectodermal disorder defined by a chronic vascularized cornea, hyperkeratotic skin lesions and sensorineural hearing loss. Due to a mutation in the *GJB2* gene. Corneal manifestations include pannus formation, leukoma, recurrent conjunctivitis and atrophy of secretory glands that can lead to LSCD.	(Cheung et al., 2019; Gicquel et al., 2002; Messmer et al., 2005)
Xeroderma pigmentosum	Hypersensitivity to ultraviolet radiation with defective DNA repair. Corneal manifestations include recurrent ulcerations, neovascularization and ocular squamous neoplasia.	(Fernandes et al., 2004)
Epidermolysis bullosa	Abnormalities of the basement membranes of the skin and mucous membranes. Most common findings include conjunctival blistering, recurrent corneal erosions, stromal scarring, pannus and neovascularization, exposure keratopathy that can lead to LSCD. Incidence of the corneal involvement depends of the subtype of epidermolysis bullosa.	(Thanos et al., 2010; Tong et al., 1999)
Autoimmune polyendocrinopathy-candidiasis–ectodermal dystrophy/ dysplasia (Multiple endocrine deficiency, APS1)	Autosomal recessive. Auto-immune reaction to ectodermal tissue, including the corneal epithelium and the limbus.	(Merenmies and Tarkkanen, 2000; Mohammadpour et al., 2006)
Dyskeratosis congenita	Syndrome of bone marrow failure secondary to unstable telomeres. LSCD from premature telomere shortening, leading to severe corneal involvement. Patients may require allogeneic hematopoietic stem cell transplant, with a risk for LSCD impaired by a graft-versus-host-disease.	(Aslan et al., 2012)

CL = contact lens; LSCs= limbal stem cells; LSCD = limbal stem cell deficiency.

Table 2.

Staging of limbal stem cell deficiency based on clinical presentation by the international consensus

Stage I: Normal corneal epithelium within the 5 mm central cornea
A: Limbal involvment < 50%
B: Limbal involvment ≥ 50% but < 100%
C: 100% of limbal involvement
Stage II: Central 5 mm of the cornea is affected
A: Limbal involvment < 50%
B: Limbal involvment ≥ 50% but < 100%
Stage III: Entire corneal surface involvment