INTRODUCTION

The cornea contains a population of self-renewing stem cells that preserve optical clarity by maintaining the integrity of the corneal epithelium. These cells, called limbal stem cells (LSCs), are primarily found at the limbus, a narrow region located at the perimeter of the cornea, directly adjacent to the conjunctiva [1, 2]. Diseases of the cornea are a major cause of blindness, and according to the World Health Organization are second only to cataract as the leading cause of blindness worldwide [3].

Ocular surface failure may result from loss of numbers or function of LSCs, and is referred to clinically as LSC deficiency (LSCD). This may be primary or secondary to a number of conditions including chemical trauma, Stevens-Johnson syndrome, contact lens-induced keratopathy, or ocular cicatricial pemphigoid [4, 5]. In these circumstances, the corneal epithelium does not regenerate and clear cornea is overtaken by bulbar conjunctival cells leading to chronic epithelial defects, inflammation, edema, neovascularization, and scarring [6]. Over time, the resulting cornea becomes opacified, and visual acuity is compromised.

LSC transplantation is key in the treatment of this disease [5–7]. If performed before the development of extensive corneal neovascularization and scarring, this may be all that is required for reasonable recovery of visual acuity. In the late stages, LSC transplantation alone may provide a stable ocular surface, but vision may be poor due to corneal neovascularization and scarring. In this situation, corneal transplantation in the form of a deep anterior lamellar keratoplasty (DALK) or penetrating keratoplasty (PK) in addition to LSC transplantation may be necessary for optimal visual rehabilitation. It is important to emphasize that a corneal transplant alone will likely fail, as the same disease process in LSCD may recur with a corneal transplant, leading to the eventual loss of optical transparency [8]. Therefore, restoring the LSCs in these patients before or alongside corneal transplantation is essential, and may prevent conjunctival invasion and potentially, graft failure. Indeed, recent work using LSC grafts, either alone or in conjunction with DALK/PK, shows promise in maintaining a clear visual axis in LSCD [5].

Despite the encouraging clinical data, the identity of the LSC has not been characterized fully, and there are no absolute specific markers that allow the prospective identification of LSCs from adjacent non-stem cells. Many of these cell types (including epithelial cells and progenitor cells) share common physical characteristics and surface molecules. Furthermore, it is uncertain whether LSCs are the only cells capable of maintaining the corneal epithelium. A number of groups now suggest that regeneration can also arise from basal epithelial cells from the central clear cornea [9–11]. In this review, we discuss classical concepts and recent advances in LSC biology and identify research and clinical challenges in corneal epithelial regeneration in ocular health and disease.

LSCs

Stem cells are defined by their ability to differentiate and self-renew in vivo, thereby contributing to the regeneration and homeostasis of living tissues. Ex vivo, they are characterized by robust proliferation, colony-forming ability, and capacity to differentiate into one or more mature cell types. The stem cells responsible for maintenance...
of the postnatal corneal epithelium are widely believed to be located at the limbus; however, this dogma has been challenged by recently published clinical observations and animal-based studies, which together propose that the central cornea may also contain these stem cells [9–11]. Early studies by Friedenwald and Buschke showed that the mitotic index of the cornea was greater toward the periphery, suggesting that most of the regenerative capacity of the cornea resided at the limbus [12]. Nearly 30 years later, Davanger and Evensen proposed that the limbal palisades of Vogt housed the cells responsible for renewal of the corneal epithelium [13]. This was confirmed by Cotsarelis et al., who identified slow-cycling, label-retaining cells (a characteristic of stem cells in the epidermis) at the limbus that can be recruited to proliferate when the cornea is damaged [1]. Importantly, these label-retaining cells were not mature epithelial cells, but were found to be immature basal cells that could differentiate into K3/K12-positive corneal epithelial cells in appropriate culture conditions and after transplantation in vivo [2]. Tseng and coworkers provided important information from clinical observations and transplantation studies that demonstrated the significance of limbal tissue in wound healing, even in cases of severe ocular surface injury, supporting previous findings that the limbus contains corneal epithelial stem cells [8, 14–16]. Using the paradigm of stem/progenitor cells found in skin epithelium (holoclone, meroclone, paraclone), Pellegrini et al. confirmed these results by performing careful clonal analysis of cells taken from biopsies from various locations on human ocular surface epithelium [17]. Altogether, these results revealed that the limbus contained the greatest concentration of stem cells in the human corneal epithelium, supporting its clinical use for ocular surface repair.

LSC ISOLATION, CULTIVATION, AND TRANSPLANTATION

LSCs are cultivated from biopsies using a variety of specialized techniques, which vary among laboratories worldwide. There have been no studies to compare the efficacy of each technique. Six protocols in particular, representing pioneers in the field with successful human transplantation results, are reviewed by Tseng et al. [18]. Typically, small biopsy specimens (1–4 mm²) are surgically removed from the limbus and then subjected to mechanical disruption (cutting/shredding) and/or enzymatic digestion (using trypsin or dispase) [6, 19–25]. Some investigators cultivate the tissue fragments directly on amniotic membrane substrate [21, 26], whereas others cultivate and expand primary cultures from explanted tissue or digested tissue suspensions before seeding onto a surgical substrate (fibrin gel, contact lens, or amniotic membrane [AM]) [5, 26, 27]. Cells are grown in either serum-containing or serum-free media, typically supplemented with epidermal growth factor (EGF) and hydrocortisone [18]. An important modification is the development of a xeno-free protocol with cultivation in autologous serum on denuded human AM [26]. Most groups cultivate primary cultures for 2–3 weeks, allowing a confluent monolayer to develop on the surgical substrate before transplantation. Some groups, including Pellegrini and coworkers, initially expand primary limbal cells by coculture with irradiated clinical-grade murine 3T3 fibroblast cell lines because it is thought that they preserve stem cell function through contact-mediated or paracrine effects [5]. Alternatively, human AM can be used for similar reasons, with experimental data suggesting that intact AM is more effective than denuded AM for maintaining stem cell states [28]. Others propose that human limbal stromal cells [29–31] or other human fibroblast lines [32, 33] can maintain LSCs while avoiding xenogeneic conditions, although work is still in the early stages. A recent report suggests that pigment epithelial-derived factor can promote LSC self-renewal in vitro, but this has yet to be confirmed by other investigators [34]. The details of surgical transplantation protocols vary among groups, but generally they involve performing a limbal peritomy and carefully removing abnormal corneal fibrovascular tissue by lamellar keratectomy [5, 21, 22]. Some groups advocate the use of mitomycin-C to prevent overgrowth of subconjunctival fibroblasts [22, 24]. Finally, a sheet of cultivated cells on selected substrate is positioned over the cornea and secured with sutures and/or fibrin glue. Postoperative care protocols also vary among groups, ranging from pressure patching and complete lid closure for 1 week [5], to no pressure patching and continuous therapeutic contact lens use for up to 3 months [22, 24]. Overall, there is no clear evidence to date to support the practice of one technique over the other for the isolation, cultivation, and transplantation of LSCs.

HETEROGENEITY OF CURRENT LSC ISOLATES

An obvious problem with current techniques is that the LSCs used for transplantation are a heterogeneous cell population because explant techniques do not use any selective measures such as immunophenotyping or fractionation. Therefore, these cultures contain many cell types, ranging from epithelial, conjunctival, or stromal stem and progenitor cells to mature conjunctival and corneal epithelial cells, goblet cells, keratinocytes, and vascular or blood cells. Importantly, the lack of stem/progenitor cell enrichment may be a cause of graft failure, as only true stem cells can contribute to long-term tissue homeostasis [4]. This is supported by post hoc analysis of clinical data showing a strong correlation between graft success and the percentage of holoclone-forming (ΔNp63α-bright) cells in initial cultures [5]. Therefore, the ability to isolate true LSCs prospectively for transplantation is crucial to ensuring successful long-term engraftment. Morphological characteristics of limbal basal cells based on histology and flow cytometry include small cell size, pigmentation, and high nuclear-cytoplasmic ratio; however, these factors have never been linked directly with LSC function [13, 35–37]. LSCs and their progeny are believed to be interspersed among the basal cells, and there is evidence to suggest that LSCs may be found in clusters at the palisades of Vogt [38]. However, harvesting a purified population of these cells remains a major challenge.

MARKERS OF LSCS

Several molecular markers of LSCs have been proposed and were reviewed by others [38–40]. Notable markers are summarized in supplemental online Table 1. The cytokeratins have been studied intensively and are generally viewed either as markers of differentiation (K3, K12), or unspecific markers of limbal basal cells (K5, K14, K15, K19). Some of these cytokeratins were also found in conjunctival epithelium (K5, K14, K19). K15 has been examined closely and deserves particular attention. K15 is a marker of hair follicle bulge stem cells, the putative epidermal stem cell, and is considered by some to be a marker for LSCs [41]. However, it is also widely expressed in limbal basal cells and has not been specifically or directly linked to LSC functions including clonogenicity or self-renewal. Vimentin stains limbal cells, but this appears
confined to transitional cells between the cornea and limbus [38], although a recent report disputes this [42]. Many of the markers associated with limbal basal cells in the past, including α9 and β1 integrins, α-enolase, and connexin-43, are widely believed to be associated with increased mitotic activity related to transient amplifying cells, and not with true quiescent stem cells [38–40, 43]. It is now generally agreed that prominent candidate markers include ΔNp63α, C/EBPα, Bmi1, ABCG2, and Notch-1 [39, 43]. Nonetheless, this remains a highly controversial area with numerous conflicting reports.

p63 is essential for squamous epithelial development and regeneration, and is expressed at high levels in both epidermal and limbal holoclones [44–46]. It is thought to regulate the stem cell population by promoting cell senescence and genomic stability, thereby sustaining their proliferative potential [47, 48]. TP63, the gene encoding p63, produces six isoforms, of which ΔNp63α is most highly associated with LSCs [44, 49]. ΔNp63α is expressed in holoclones (stem cells) but not paracolones (transient amplifying cells) in quiescent cornea, and its expression increases in limbal basal cells during wound healing [48]. This process appears related to C/EBPα inactivation [48]. Importantly, greater ΔNp63α staining is associated with greater success in clinical LSC transplantation, whereas grafts containing <3% of ΔNp63α-bright cells are at an increased risk for failure [5].

C/EBPα and Bmi1, like ΔNp63α, are expressed in holoclones but not paracolones [43]. C/EBPα is found in many epithelial cell types and is involved in cell cycle arrest [48]. Bmi1 is a polycomb group repressor initially discovered to be important for self-renewal of hematopoietic stem cells, and later found to be expressed in LSC-associated limbal side-population cells by flow cytometry [50, 51]. Both C/EBPα and Bmi1 are thought to regulate LSC senescence, and the expression of both factors becomes downregulated in response to corneal injury. LSCs continue to express ΔNp63α during the initial proliferative phase, but lose this expression on terminal differentiation [48]. The expression of all three factors is currently the most widely accepted definition of an LSC.

ABCG2, an ATP-binding cassette transporter protein, is a cell surface protein that can be detected based on cellular efflux of Hoechst dye on flow cytometry and is the basis for the so-called side-population cells. It is a characteristic associated with stem and progenitor cells in many tissues (including blood and skeletal muscle) and can be detected reliably both histologically and on live cells, permitting its use for cell purification strategies [52, 53]. However, it is not entirely specific on its own and strategies to isolate stem cells use it with a number of other positive or negative markers to achieve sufficient cell purity. ABCG2 is expressed by only a small subset of limbal basal cells (0.4%), but interestingly was not associated with extensive colony-forming capacity [51]. Notch-1 has been identified in a subset of ABCG2-expressing cells, colocalizing mostly with limbal basal cells in the palisades of Vogt [54]. It seems to be highly expressed in quiescent cells, supporting the notion that it could be an LSC marker [54].

ARE LSCs THE EXCLUSIVE CORNEAL EPITHELIAL STEM CELL?

Despite decades of work supporting the existence of the LSC and its crucial role in corneal epithelial homeostasis and wound repair, the current view has been generated mostly from observational and clinical studies. This dogma was recently challenged and is the subject of intense debate. In 2008, Majo et al. [10] published a landmark study in which they performed central corneal transplants from transgenic mouse donors constitutively expressing either green fluorescence protein or β-galactosidase. When explants (grafted onto the limbus of recipient nude mice) were challenged with central epithelial wounding, labeled cells contributed significantly to the healed epithelium, even after serial transplants, suggesting that long-term stem cell activity exists in the mouse central cornea [10]. This work is supported by clinical observations. Dua et al. [9] examined clear central corneal islands from eight eyes of five patients with a confirmed diagnosis of LSC deficiency. They revealed that despite the apparent lack of LSCs, the central cornea remained normal in appearance throughout the follow-up period (8–12 years), again suggesting that the central cornea has the ability to maintain normal corneal epithelium [9]. Moreover, given the extended length of follow-up, the maintenance of a clear cornea in their patients is not likely a result of a transient progenitor population. Bi et al. recently described the case of a patient with monocular vision with Terrien marginal degeneration and a perforated cornea, who maintained a clear central cornea throughout the follow-up period (32 months) despite having received 360 degrees of ring-shaped glycercin-cryopreserved lamellar keratoplasty over two operations [11]. Bi et al. also show the healing of a central corneal epithelial defect despite the apparent lack of viable LSCs [11]. Indeed these reports are rare and the source of much controversy and debate in the field, but together they highlight the need for further studies to prospectively identify, isolate, and characterize corneal epithelial stem cells.

CONCLUSION

Our accumulated knowledge suggests that the vast majority of corneal epithelial stem cells reside at the limbus, and that
replacement of these cells represents a viable and emerging op-
tion for the treatment of ocular surface failure secondary to LSCD. 
Indeed, there are many notable reports of clinical success at var-
ious centers worldwide with LSC transplantation despite gaps in 
our current understanding and low efficiency for the isolation of 
LSCs with true stem cell characteristics. Current strategies are la-
bror intensive and lack standardization, in large part because it is 
currently impossible to isolate pure, standardized populations of 
these cells prospectively for research or clinical use. Until this 
occurs, different centers worldwide will likely use the specific 
techniques for isolation and ex vivo culture of LSCs in their respec-
tive institutions that have been studied and developed in their 
basic laboratories. Comparative studies may still be helpful to de-
termine which of these current techniques may be more clinically 
successful before the emergence of new methods to isolate, 
grow, and transplant the optimal regenerative cells. In light of 
this, several questions will continue to surface. Which limbal cells 
are best suited for therapy, and how can we purify them? Which 
growth conditions can best maintain their stem cell qualities of 
self-renewal? Where exactly are these cells located, and will recapitulation of their niche during ex vivo 
expansion contribute to better clinical outcomes? Can we ma-
nipulate LSCs with medications? Can these cells be used to de-

civer therapeutics? These are all questions with far-reaching 
implications, which, like many other fields in regenerative med-
icine, will be best solved through open collaboration between 
scientists and clinicians.

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AUTHOR CONTRIBUTIONS

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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