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Strategies for Reconstructing the Limbal Stem Cell Niche

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Abstract

The epithelial cell layer that covers the surface of the cornea provides a protective barrier while maintaining corneal transparency. The rapid and effective turnover of these epithelial cells depends, in part, on the limbal epithelial stem cells (LESCs) located in a specialized microenvironment known as the limbal niche. Many disorders affecting the regeneration of the corneal epithelium are related to deficiency and/or dysfunction of LESCs and the limbal niche. Current approaches for regenerating the corneal epithelium following significant injuries such as burns and inflammatory attacks are primarily aimed at repopulating the LESCs. This review summarizes and assesses the clinical feasibility and efficacy of current and emerging approaches for reconstruction of the limbal niche. In particular, the application of mesenchymal stem cells along with appropriate biological scaffolds appear to be promising strategies for long-term revitalization of the limbal niche.

Keywords

Corneal Limbus; Stem Cell Niche; Epithelial Cell; Limbal Epithelial Stem Cell Deficiency; Regenerative Medicine; Mesenchymal Stem Cell; Extracellular Matrix

1. Introduction

The corneal epithelium provides a protective barrier while serving a critical role in maintaining corneal transparency. It is continuously turned over as the most superficial cells of the corneal epithelium are shed from the surface and are replenished by the limbal epithelial stem cells (LESCs). LESCs derive their name from their anatomic origin in the boundary between the cornea and conjunctiva called the limbus [1]. The proliferation, migration, and differentiation of LESCs are dependent upon their specialized microenvironment known as the limbal niche. The limbal niche is characterized by unique

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physical and chemical features that include cells (e.g., mesenchymal cells, immune cells, melanocytes, vascular cells, and nerve cells), extracellular matrix (ECM), and signaling molecules (e.g., growth factors and cytokines) [2-7]. Significant pathology involving any component of the limbal niche can result in LESC dysfunction that contributes to effective limbal stem cell deficiency (LSCD) [6, 8, 9]. Accordingly, the primary aim of this review is to discuss current and emerging strategies for treating LSCD with a focus on reconstruction of the limbal niche.

2. The Limbal Niche

2.1. Microenvironment Structure

Recent developments in imaging technologies and the use of cellular and molecular analysis tools have led to an improved understanding of the limbal microenvironment (Figure 1) [5, 10-14]. The limbal niche contains ridges, known as the palisades of Vogt, that correspond to undulations of the epithelium and the stroma. Specifically, the epithelium extends more deeply in the limbal area and is marked by the intervening stromal areas that appear as lines on clinical examination (Palisades). The basal layer of the limbal epithelium in these areas, which anatomically have also been called limbal epithelial crypts (LECs), harbor the LSCs [14-16]; although, LSCs have not been found in every LEC, and distinct distribution patterns of LSCs have been identified in different individuals [3, 11, 15]. These microenvironments have unique gene expression and ECM protein profiles that are specifically suited to, and critical in the maintenance and function of LSCs [12-14]. Also, the limbal niche is populated by numerous cell types including melanocytes [17], immune cells [12], vascular cells [16], nerve cells [18], and stromal (mesenchymal) cells [19]. Mesenchymal stem cells (MSCs) have garnered increased attention in recent years due to their role in LESC regulation. Mesenchymal CD90 and CD105 positive cells locate preferentially in LEC areas, and have been shown to have close interactions with LSCs (Figure 2) [5, 10, 11, 20]. In vivo confocal microscopy (IVCM) has identified clusters of hyper-reflective mesenchymal cells in the anterior limbal niche stroma subjacent to the basal epithelium, where LSCs are located [20]. Beyond anatomic proximity, mesenchymal cells have been shown to interface with LSCs through a number of molecular substrates and signaling pathways that include aquaporin-1 and vimentin [21], chondroitin sulfate (6C3 motif) [10], SDF-1/CXCR4 [22], BMP/Wnt [23], and IL-6/STAT3 [24]. Additional mechanisms of interaction include intercellular contact and the secretion of paracrine growth factors [25, 26], and complementary effects on cytokine and growth factor expression [27]. Vimentin-positive sphere forming cells isolated from human limbal tissue have been likewise been shown experimentally to reconstruct the limbal niche and re-cellularize decellularized human corneas in vitro [28]. This information suggests that, in comparison to other limbal niche cells, MSCs may be uniquely critical to LESC function. This finding is consistent with the role of MSCs in different stem cell niches throughout the body [29].

2.2. Pathological Alterations

Significant corneal injury can perturb the limbal niche and lead to LESC dysfunction due to tissue destruction and inflammation. All cases of LSCD consistently reveal various degrees of inflammation [30-32], and are characterized by multiple deleterious molecular and

cellular changes within the cornea and limbus. The ocular surface of conjunctivalized corneas demonstrates increased levels of inflammatory and angiogenic mediators including IL-1 α , IL-1 β , IL-1 RA, IL-6, VEGF, ICAM-1, and VCAM-1 [33]. Pathophysiologically, persistent inflammation appears to contribute to LESC dysfunction through multiple mechanisms. Under normal circumstances, an ocular surface insult triggers migration of innate and adaptive immune cells to the site of injury. The action of these recruited cells in conjunction with resident cells facilitates the resolution of tissue damage through the stimulation of LESC proliferation and differentiation [34]. Alternatively, under pathologic conditions, the persistence of a pro-inflammatory environment results in continued secretion of inflammatory cytokines (interferon gamma, etc.), impaired macrophage phagocytosis, pathologic stimulation of T-lymphocytes, and (hem/lymph)angiogenesis [8, 35-37]. Furthermore, LESC, during these inflammatory conditions, exhibit decreased expression of stem cell markers and reduced colony forming efficiency [6, 8]. Lastly, inflammation can result in limbal niche alterations including changes in the extracellular matrix (ECM) and adhesive molecules, decreased expression of S100 proteins, and atypical changes in the density and morphology [6, 12, 38].

Given the importance of the limbal microenvironment to LESC, significant distortions in the former will invariably result in dysfunction of the latter. As such, a two-pronged approach to management is necessary for LSCD. In addition to direct replacement of the lost LESC population, restoration of the limbal niche is critically important in order to restore a suitable LESC microenvironment. Indeed, successful strategies for restoration of the ocular surface in this setting require treatment of both limbal stem cell deficiency and etiologies of dysfunction. Several strategies have been proposed and assessed throughout the last decade for repopulating the LESC and revitalizing the limbal niche (Figure 3). However, these strategies are in various stages of development and many have not reached the clinic.

3. Repopulation of Limbal Epithelial Stem Cells

3.1. Limbal Transplantation

Limbal transplantation is a surgical procedure where limbal tissue is transplanted to an eye with LSCD. It replaces both the limbal niche and the LESC at the same time. The current medical and surgical management of LSCD depends on the severity and the extent of LSCD (e.g., partial or total, unilateral or bilateral). In patients with symptom-free partial LSCD, expectant management with restoration of the tear film and suppression of inflammation is recommended [9]. However, in symptomatic partial LSCDs, in addition to anti-inflammatory therapy, surgeries such as amniotic membrane transplantation (AMT) [39], sequential sector conjunctival epitheliectomy [40], and ipsilateral limbal translocation onto the stem cell-deficient areas may be considered [41].

The management of total LSCD is challenging and often requires major surgical interventions. In cases of unilateral total LSCD, conjunctival limbal autograft (CLAU) from the healthy eye has been performed successfully for the last 3 decades [42, 43]. A newer surgical method, simple limbal epithelial transplantation (SLET), has been developed to reduce the risk for injuring the fellow (donor) eye. In this technique, a small (2×2 mm) limbal tissue is harvested from the fellow eye and cut into smaller pieces and grafted onto

different areas of the recipient cornea using an amniotic membrane. The mid-term results of SLET are promising [44-46].

In bilateral total LSCD, the main treatment option is transplantation of allogeneic limbal grafts from cadaver eyes or healthy donors. For example, in living related-conjunctival limbal allograft (lr-CLAL), the limbal tissue and adjacent conjunctiva are harvested from a living related donor [42, 43, 47]. In comparison, limbal tissue with adjacent corneal tissue is harvested from cadaver eyes in keratolimbal allograft (KLAL) [47]. The overall success rate for limbal grafts has been reported from 33% to 77% in published studies depending on the underlying etiology [48]. The major drawback in allogeneic keratolimbal or conjunctivallimbal transplantation is the requirement for long-term systemic immunosuppression in the recipient [49].

An interesting finding in patients who have undergone limbal allograft transplantation is the presence of both host and donor epithelial cells at long-term follow-ups [50]. This finding may indicate the possibility of regeneration or reactivation of the limbal stem cell niche after limbal allograft transplantation. Although donor limbal grafts play a significant role in repairing the corneal epithelium, the presence of the recipient epithelial cells reinforces the hypothesis that by revitalizing the recipient limbal niche, the host LSCs can be reactivated to differentiate and re-populate the corneal epithelium.

3.2. Cultivated Limbal Epithelial Cells

To minimize the potential for complications to the donor eye in limbal transplantation (in cases of autologous or living-related limbal tissue harvest), the use of ex-vivo cultivated limbal epithelial cells has been pursued [49, 50]. In ex-vivo cultivated limbal epithelial transplantation (CLET), a small piece of limbal tissue is harvested from the donor eye, and the epithelial cells are expanded in culture with or without feeder cells [51, 52]. The use of feeder cells, although not completely necessary, actually provides a suitable microenvironment, similar to limbal niche, for self-renewal and maintenance of LSCs. Irradiated or mitomycin-treated murine 3T3 cells (mitotically inactive) have been used as the prototypic feeder cells [51, 52]. More recently, feeder-free culture systems, as well as alternative feeder cells such as MSCs and limbal melanocytes from human sources, have been established [26, 53]. Moreover, substituting xenogeneic cell culture products (e.g., serums and growth factors) with human or recombinant materials, or developing serum-free cell cultivation systems, have diminished the risk of transmitting animal-derived infections [54].

After satisfactory expansion in culture, limbal epithelial cells are transplanted using cell carriers. Human amniotic membrane (HAM) has been used as the carrier in most in vivo studies and clinical trials [55-57]. Nevertheless, batch to batch variability, a possible risk for disease transmission and the low transparency of HAM have led to the search for new carriers. Besides HAM [58], a wide range of natural or synthetic biomaterials have been proposed as carriers for cultivated LSCs, such as collagens (hydrogels, cross-linked, or compressed) [59-61], fibrin [62], and siloxane hydrogel contact lenses [63]. Most of these biomaterial scaffolds to date have only been studied in animal models with no human data on their safety and efficacy. Nonetheless, the fabrication of new biomaterials and cell

carriers is expected to further expand the use of cell-based therapies for corneal regeneration in the future.

3.3. Non-Limbal Epithelial Cells

The shortage of allogeneic limbal tissues, the necessity for systemic immunosuppression and the risk of disease transmission have led to an interest in finding alternative autologous sources of epithelial cells for restoring bilateral total LSCD. In the last decade, a number of alternate sources have been introduced, including oral mucosal epithelial cells [64, 65], cultivated autologous conjunctival epithelial cells [66-68], or epithelial-like cells differentiated from pluripotent and/or multipotent stem cells including amniotic epithelial cells [55], embryonic stem cells [69], induced pluripotent stem cells (iPSCs) [70-73], hair follicle bulge-derived epithelial stem cells [74], umbilical-cord lining epithelial stem cells [75], mesenchymal stem cells [19, 76], and human immature dental pulp stem cells [77]. Cultivated oral mucosal epithelial transplantation (COMET) has already been used in clinical studies in the last decade. The clinical results of COMET showed an average success rate of 72%, particularly for achieving ocular surface stability. However, the phenotype of the transplanted epithelium remains oral mucosal and does not appear to change to corneal, hence given the thicker and more opaque epithelium, the visual outcomes are less than optimal [78, 79].

Another autologous source is conjunctival epithelial cells (CjECs) derived from patients. Preliminary studies have demonstrated that ex-vivo cultured human CjECs on HAM can produce five to six epithelial layers following in vitro (air-lifting) cultivation express cytokeratin 4, 13, 3 and MUC4 [66, 68]. The ex vivo HAM-cultivated autologous CjECs were transplanted onto the eyes of patients with LSCD with a reported success rate of 86% after an average follow up of 18.5 months with decreased corneal opacification, conjunctivalization, and epithelium breakdown [67]. IVCN also demonstrated well-formed five to six-layered epithelium with regular hexagonal basal cells [67].

Currently, the long-term results of COMET and CjECs transplantation are not available. Oral mucosal epithelial cells and conjunctival epithelial cells do not possess the same functionality as corneal epithelial cells. Their transplantation may primarily lead to surface stabilization as there is no evidence of limbal niche regeneration following COMET or CjECs transplantation. Thus, corneal epithelial cells derived from pluripotent or multipotent cells (e.g., iPSCs) have been pursued [70-73]. The most recent report indicates that epithelial cells isolated from ectoderm zone of a human iPSCs-derived self-formed ectodermal autonomous multi-zone can regenerate the ocular surface in a corneal epithelial stem cell deficiency model [80]. However, epithelial-like cells derived from multipotent or pluripotent stem cells are only in the preliminary stages of research, and have important practical (e.g., teratoma formation) and ethical concerns.

4. Reconstruction of the Limbal Stem Cell Niche

A number of strategies for repopulating the limbal epithelial cells and restoring the niche have been proposed (Figure 3). Previous studies have indicated that in severe cases of LSCD, the simple administration of limbal epithelial stem cells may not be sufficient for the

longterm rehabilitation of the ocular surface [45, 46, 50, 57, 81, 82]. In particular, epithelial stem cells transplanted to a hostile ocular surface environment where a healthy stem cell niche cannot be re-established, are likely to be lost over time. As mentioned above, chronic inflammation and dysfunction of resident cells and the ECM are the major causes of limbal niche disturbance after insults. Thus, strategies for reconstructing the limbal stem cell niche are focused on decreasing inflammation and restoring proper function of the resident cells and ECM [9]. Some of these strategies include the administration of scaffolds/matrices, hemoderivatives, and mesenchymal stem cells.

4.1. Bio-active Extracellular Matrix for Limbal Niche Replacement

As mentioned above, the ECM plays a crucial role in the function of the limbal niche. Therefore, a successful strategy for reconstructing the limbal niche most likely should involve the use of a regenerative ECM.

Amniotic Membrane—Nowadays, the most popular scaffold for ocular surface reconstruction is human amniotic membrane. Amniotic membrane (AM) is the innermost layer of placental membranes surrounding the fetus. This non-vascularized and non-innervated tissue is composed of five layers. The amniotic epithelial cells (AECs) and amniotic mesenchymal cells (AMCs) are located in the epithelial and fibroblastic layers of the AM, respectively, and produce cytokines and growth factors that become imbedded in the AM ECM [83, 84]. The ECM of AM contains collagen types I, III, IV and V, laminin 1 and 5, and fibronectin and various growth factors/cytokines such as epidermal growth factor (EGF), and hepatocyte growth factor (HGF) [85].

The collagen-rich structure of AM makes it suitable as a scaffold in cell delivery approaches as well as tissue engineering [86]. Thus, AM has served as a proper scaffold for cultivation and delivery of LSCs [58]. It has been suggested that the AM matrix can provide a niche like environment for LSCs [87]. More recently, HC-HA/PTX3 has been purified from cryopreserved AMs and is proposed as the responsible element for preserving the LSCs quiescence by upregulating BMP signaling [88].

Although AM provides a proper ECM with anti-inflammatory properties for supporting and reconstructing the ocular surface and limbal niche, there are some shortcomings. The AM is an opaque tissue with low tensile strength with batch to batch variability [89, 90], that also carries a small risk of infectious disease transmission. More importantly, since AM is typically digested after transplantation, its benefits may be more short-lived and its long-term contribution to the limbal niche ECM may be limited [39]. Therefore, alternatives to AM that can address these concerns are desirable.

Fabricating bio-active ECMs for Niche reconstruction—As mentioned above, the limbal niche is a three-dimensional structure constructed with an integrated ECM. Therefore, replenishing a regenerative fabricated ECM could be a potential strategy to restore the function of the limbal niche. The protocols for producing bio-active ECMs generally depend on using purified/recombinant structural proteins such as collagen or decellularization of animal or human corneas. Limbal crypts have been fabricated using type I collagen and cast molding. These bioengineered crypts supported the proliferation and

phenotype of human LESC in addition to providing the appropriate structure for alignment at the edge of created crypts [91]. Likewise, a novel approach for fabricating cell-laden corneal/limbal constructs is to perform three-dimensional printing using a mixture of collagen, elastin and laminin as bio-ink [92, 93].

Decellularization of porcine and human corneas has been studied as another approach for producing a bio-active ECM [94, 95]. These protocols rely on the application of detergents, osmotic solutions, and ribonucleases to remove all cellular components and to reduce antigenicity. The bio-activity of decellularized corneas has been shown by cultivating the corneal epithelial cells on the prepared matrix/scaffolds and also transplantation in animal models [94-97]. Moreover, some clinical studies have investigated the results of decellularized porcine cornea transplantation in cases with corneal ulcerations [98, 99]. This approach is mostly applicable to situations requiring stromal replacement with healthy epithelium and thus its application to LSCD may be limited. Therefore, an alternative protocol to fabricate a bio-active ECM has been proposed by digesting the decellularized corneas and producing a hydrogel. The fabricated hydrogel provided proper support for in vitro culture of corneal stromal cells [100, 101]. Producing a bio-active ECM hydrogel from decellularized corneas could potentially be an effective strategy for reconstructing the limbal niche. All in all, the general concept should consist of a bio-active ECM containing structural proteins as well as healing factors.

4.2. Biological Factors to Revitalize the Limbal Niche

As noted earlier, the function of the limbal niche is highly dependent on the proper signaling and communication between its cellular components. While most of the critical signaling factors remain to be identified, local delivery of exogenous growth factors is an attractive approach for restoring the function of the niche.

Hemoderived Factors—Blood-derived factors are currently used widely in the clinical setting. Serum or plasma-derived eye drops contain similar growth factors, cytokines, vitamins and minerals as tears for supporting corneal epithelial homeostasis, proliferation, and differentiation [102]. Therefore, these products such as autologous/allogeneic serum eye-drops (ASE), and platelet-derived preparations are favorable options for revitalizing the limbal stem cell niche. Autologous serum eye-drops have been shown to contain EGF, TGF- β , fibronectin, vitamin A and other types of cytokines and support factors required for normal homeostasis of limbal and corneal epithelium [103-105]. Clinical studies have shown successful rehabilitation of the ocular surface after administration of ASE for patients with persistent epithelial defect following LSCDs [106, 107]. It has been found that administration of ASE (diluted or not), results in restoration of a healthier ocular surface; thus, it has been used in severe conditions such as graft-versus-host disease, dry eye disease, Sjogren disease and keratoconjunctivitis [103, 106, 107].

Three types of preparations from platelets have been introduced including platelet releasate (PR) [108], plasma rich in growth factors (PRGF) [109], and platelet-rich plasma (PRP) [102]. All products are obtained from the supernatant of anti-coagulated whole blood with some differences in preparation protocols. Platelet-derived preparations are rich in growth

factors (e.g., EGF, TGF, PDEF, bFGF, and IGF-1), which are potentially useful in the regeneration of the limbal niche. Animal and clinical studies demonstrated compatibility, regenerative capacity and reconstructive effects of platelet-derived preparations [102], despite some differences in the reported efficacy possibly related to different preparation methods used [110].

Bio-active Soluble Factors/Cocktails—Soluble bio-active factors/cocktails derived from various sources have been investigated for regenerating the ocular surface and limbal niche. These factors have been purified from human tissues [111], cell secretomes [76, 112-114], and/or produced by recombinant techniques [115, 116].

Amniotic membrane extract eye drop (AMEED) has been produced from HAM by homogenizing the tissue and collecting the supernatant following centrifugation. The resultant AMEED is a cocktail of AM soluble factors, which was reported to enhance the in vivo cultivation of limbal stem cells in patients with LSCD [117]. Moreover, HC-HA/PTX3 has been purified from HAM as a potential soluble factor for regeneration of the limbal niche. It is composed of heavy chain 1 (HC1) of inter- α -trypsin inhibitor covalently bond with hyaluronan (HA) and associated with pentraxin 3 (PTX3) [111]. It was shown that HC-HA/PTX3 complex supports the self-renewal of limbal niche cells and LSCs via the modulation of Wnt/BMP signaling in three-dimensional culture systems [88].

Another soluble growth factor that has been extracted from human plasma to regenerate the ocular surface is pigment epithelial-derived factor (PEDF). PEDF and its derivatives have been found to promote self-renewal of limbal epithelial stem cells in vitro. 44-mer PEDF accelerated the proliferation of LSCs via activating the p38 MAPK and STAT3 pathways [118]. The in vivo application of 44-mer PEDF in animal models of corneal epithelial injury also resulted in the restoration of limbal niche anatomy and function [118]. Other factors that have shown regenerative potentials in limbal epithelial stem cell deficiency/dysfunction are ciliary neurotrophic factor and an IL-1 receptor antagonist peptide [115, 116].

Secretomes (or condition media) is the supernatant of in vitro cultivated cells that contains all the secreted factors by those cells. In particular, the secretome of mesenchymal stem cells includes a combination of healing factors/cocktails useful in revitalizing the limbal niche and the ocular surface [76, 113, 114]. Condition media from limbal mesenchymal stem cells improves wound healing and decrease angiogenesis which is mostly mediated by soluble fms-like tyrosine kinase-1 (sFLT-1) and PEDF [113]. Moreover, the limbal MSC secretome modulates the immunophenotype and angiogenic function of macrophages to diminish their pathologic role in developing corneal neovascularization [114].

Exosomes are ultra-microscopic vesicles responsible for cell-cell communications and contain growth factors, ribonucleotides, and transcription factors. It has been shown that corneal MSCs-derived exosomes are uptaken by corneal epithelial cells in vitro and in vivo and promoted wound healing in animal models [119].

4.3. Cell-Based Approaches For Restoring the Limbal Niche

Mesenchymal stem cells—Mesenchymal stem cells (MSCs) have attracted considerable attention for the reconstruction of the ocular surface and limbal niche in recent years. MSCs were first isolated from bone marrow aspirates in 1968 by Fridenstein and his colleagues [120]. Fridenstein observed that some adherent fibroblastic cells have the potential for regenerating bone defects. Further studies also revealed that these cells have the potential for reconstruction of damaged tissues [120]. One of the critical characteristics of MSCs, which makes them suitable for usage in organ transplantation and patients with autoimmune disorders, is their immunomodulatory properties [121]. MSCs also have the ability of producing an extracellular matrix in 3-D culture systems [122, 123]. The International Society for Cellular Therapy (ISCT) has established that the minimum criteria that characterize human MSCs are (1) plastic adherence, (2) differentiation to adipocytes, chondrocytes, and osteocytes, and (3) positive expression of CD105, CD73, and CD90, and negative expression of CD14, CD34, CD45 and HLA-DR [124]. In this section, we will discuss these properties of MSCs in detail. Recently, a number of studies have focused on the administration of MSCs from various sources in animal models of ocular surface disorders, including chemical burns [125, 126], dry eye syndrome [127], limbal stem cell deficiencies [76], and corneal transplantation [128]. Among published articles, MSCs derived from bone marrow (BM-MSCs) [129], adipose tissue (AD-MSCs) [130], HAM [56], omentum [131], or limbus [76, 132], have been studied in the reconstruction of corneal surfaces (Table 1). It appears that the paracrine effect of BM-MSCs improves the survival and function (EGF expression) of limbal epithelial cells in vitro [133]. For example, native BM-MSCs migrate from bone marrow to inflamed cornea due to increased SDF-1 and substance-P in damaged cornea and peripheral blood following thermal corneal injury [134]. The increased expressions of anti-inflammatory cytokines including transforming growth factor- β (TGF- β) and interleukin-1Ra (IL-1Ra) following localization of BM-MSCs on the damaged cornea, can lead to significant regeneration of the corneal epithelium [134]. It has also been shown that bone marrow-derived MSCs can attenuate the expression of immunomodulatory molecules and cytokines (TNF- α and INF- γ) stimulated by injured corneal epithelial cells [135]. BM-MSCs can also secrete beneficial soluble factors for reconstructing the limbal microenvironment, e.g., epidermal growth factor (EGF) [136]. It has also been shown that BM-MSCs delivered on the chemically injured ocular surface can reduce the mediators of lipid peroxidation and oxidative stress, which further lead to decreased numbers of apoptotic cells and pro-inflammatory cytokines, e.g., IL- β , IL-2, and INF- γ , and attenuated corneal neovascularization [126, 137, 138].

Similarly, results of studies on limbal-mesenchymal stem cells (L-MSCs, also known as limbal stromal cells or limbal fibroblasts) appear to be encouraging. L-MSCs are derived from limbal tissues [139-141]. And as mentioned earlier, appear to have a major role in the maintenance of limbal stem cell niche [53, 139, 140]. L-MSCs are in close contact with limbal epithelial stem cells (Figures 1 and 2) [11, 22, 25, 140]. These cells can be isolated from limbus either by enzymatic digestion or by explant techniques [25, 76]. It has been shown that the L-MSCs have very similar properties and gene expression pattern as bone marrow-derived MSCs [27, 142]. They meet the criteria established by the ISCT for mesenchymal cell therapy [139]. L-MSCs are CD105⁺, CD73⁺, and CD90⁺, and also

CD14⁻, CD34⁻, CD45⁻ and HLA-DR⁻. In addition, the L-MSCs express stem cell markers including Nanog, Oct-4, Sox-2, Rex-1, and SSEA-4,[140] and were successfully differentiated into adipocytes, chondrocytes, and osteocytes [27]. Moreover, L-MSCs produce sphere colonies in three-dimensional (3D) culture conditions. The 3D cultures of L-MSCs lead to increased expression of stem cell markers, which are crucial for maintaining limbal epithelial progenitor cells in an undifferentiated state in vitro [140]. L-MSCs also have similar immunomodulatory properties to other MSCs and inhibit immune cells including T-cells in vitro [143]. Topical or sub-conjunctival administration of L-MSCs in rat alkali-burn animal models results in decreased corneal opacification; attenuated corneal neovascularization and improved fluorescein staining results [132]. As mentioned above, it seems that MSCs are likely to support the restoration of the limbal niche by secreting regenerative factors rather than direct contribution to the tissue structure.

5. Clinical Feasibility and Efficacy of Summarized Strategies for Limbal cell repopulation and Limbal Niche Reconstruction

Here, we have summarized the most recent published methods for repopulation of the LESC and regeneration of limbal niche following LSCD. The current evidence for each strategy has been provided in Table 2 and the strength of each method is graded based on current published literature and expert opinion. The strategies for repopulation of LESC are more clinically advanced today than methods of limbal niche reconstruction. However, the failures of the former have resulted in realizing the necessity for advancing the latter. Comprehensive assessment of LESC repopulation strategies has done elsewhere [144, 145].

Although blood-derived products are standard procedures for managing mild to moderate ocular surface instabilities, their efficacy in severe epithelial disease is likely limited. Similarly, the application of HAM in extensive conjunctivalization is limited [146], as it cannot provide a long-term restoration of the corneal phenotype [39]. Therefore, despite the presence of beneficial factors in HAM and blood-derived products, more advanced approaches are necessary to provide more effective therapies for severe cases.

Administration of the MSCs is an emerging strategy for the management of severe ocular surface disease. It is hypothesized that MSCs' therapeutic effects are largely due to their immunomodulatory factors which secondarily help restore the limbal niche. The safety and efficacy of bone-marrow MSCs have been investigated in several non-ocular clinical trials; however, despite many in vivo animal studies (Table 1) in ocular surface injuries, clinical testing has been limited.

6. Summary and Conclusion

Physiologic homeostasis of the LESC including their self-renewal, differentiation, and migration depends on a functional stem cell niche. The limbal niche is composed of a specialized ECM, signaling molecules as well as niche cells, which can become dysfunctional or destroyed in certain hereditary conditions or severe insults to the limbus. Reconstruction of the limbal stem cell niche is necessary for long-term restoration of the function of the LESC. In addition to transplantation-based techniques which are already in

use clinically, current regenerative approaches involve the use of biologic or synthetic scaffolds, along with hemoderivatives, cytokines or growth factors. Likewise, besides epithelial-based cell therapy approaches, mesenchymal stem cells, with their potent immunomodulatory properties and ability to produce trophic and ECM factors to support LESC, currently offer a promising candidate for cell-based therapy for restoring the limbal niche. Nevertheless, advanced clinical trials in addition to more investigations in vitro and in vivo are crucial to establishing clinical feasibility in the future.

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Figure 1:

Schematic of the limbal niche. The corneoscleral limbus contains the Palisades of Vogt (PV) and limbal epithelial crypts (LECs). The limbal epithelial stem cells (LESCs) are in close contact with niche cells including melanocytes and mesenchymal stem cells (MSCs) in the LECs. The basement membrane of the cornea, limbus, and conjunctiva have different constructs which are in turn necessary for maintaining proper homeostasis. In the basal epithelial layer of LEC, the LESCs are divided symmetrically into two identical cells (in the horizontal plane) or asymmetrically to give rise to another LESC and a transient amplifying cell (TAC, in both vertical and horizontal planes). Then, the TACs are divided into postmitotic cells (PMCs) as they migrate centripetally, Y. The PMCs are then differentiated into terminally differentiated cells (TDCs) and shed from the corneal surface, Z. In physiological situations, the sum of X and Y is equal to Z. Abbreviations: LESC, Limbal epithelial stem cell, TAC, Transient amplifying cell, PMC, Post-mitotic cell, TDC, terminal differentiated cell, MSC, mesenchymal stem cell.

Figure 2:

Schematic illustration of the interaction between limbal MSCs with LSCs in the limbal niche. In situ microscopic evaluations and specific staining have shown the existence of a physical cross-talk between limbal MSCs and LSCs. The limbal MSCs attach to the basement membrane by integrin $\alpha 8$ and dystroglycan. Moreover, MSCs projections pass through the basement membrane and have direct contact with LSCs [11, 12].

Figure 3: Diagram summarizing current strategies and materials for restoring the function of the limbal stem cells in limbal stem cell deficiency/dysfunction. The current stage of clinical development is also included. Abbreviations: CLAU, Conjunctival limbal autograft, KLAL, Kerato-limbal allograft, Ir-CLAL, Living related conjunctival limbal allograft, SLET, Simple limbal epithelial transplantation, COMET, Cultivated oral mucosal epithelial transplantation, AET, Amniotic epithelial transplantation, CJET, Conjunctival epithelial transplantation, HAM, Human amniotic membrane.

Table 1.

Summary of studies evaluated the effects of Mesenchymal Stem Cells (MSCs) from different sources on corneal injury/disease animal models.

Source of MSCs	Host Animal	Disease Model	Route of Administration	Carrier/Scaffold	Brief Results and Proposed Reconstructing Mechanisms	Ref
Human BM	Rat	Alkali burn	Corneal surface	Denuded HAM	Applied cells survived. ↓ neovascularization, ↓ inflammation (↓ CD45, ↓ IL-2 and ↓ MMP-2), No differentiation to epithelial cells	[137]
Human BM	Rat	Alkali burn	Corneal surface	AM	BM-MSCs pre-differentiated to corneal epithelial lineage. ↑ Expression of K3 and p63 after transplantation	[147]
Human BM and Mouse BM	Murine	Dry Eye Syndrome	Periorbital injection	-	↓ CD4 ⁺ T cell infiltration, proliferation and differentiation, ↓ inflammatory cytokines (↓ IL-2 and ↓ IFN-γ), ↑ aqueous tear production, ↑ conjunctival goblet cells	[127]
Mouse BM	Mouse	Alkali burn	Intravenous	-	MSCs were pre-treated with INF-γ. MSCs homed in the injured cornea, ↓ infiltration of immune cells, ↓ pro-inflammatory cytokines (↓ IL-1α, ↓ IL-6, and ↓ NO)	[148]
Mouse BM	Mouse	Thermal Cautery Injury	Intravenous	-	↑ Native and injected BM-MSCs in the damaged cornea (not normal cornea), ↑ anti-inflammatory cytokines (↑ IL-1Ra and ↑ TGF-β), Reconstruction of the corneal epithelium	[134]
Mouse BM	Mouse	Alkali burn	Corneal surface	-	↓ Corneal epithelial defects, ↑ K3/12 ⁺ cells	[149]
Rat BM	Rat	Alkali burn	Corneal surface	HAM	Before transplantation: BM-MSCs induced by rat-CSCs co-culture, ↑ expression of K12, ↑ similar corneal epithelial cell morphology. After transplantation: ↓ opacity, ↓ neovascularization, ↓ fluorescence staining area	[150]
Rat BM	Rat	Alkali burn	Corneal surface	Polysaccharide hydrogel	Corneal epithelium repaired, ↓ corneal opacity, ↓ neovascularization, ↑ anti-inflammatory and ↑ anti-angiogenic cytokines (↑ TGF-β and ↑ TSP-1), ↓ inflammatory (↓ TNF-α), ↓ chemotaxis (↓ MIP-1α and ↓ MCP-1), and ↓ angiogenesis (↓ VEGF and ↓ MMP-2) factors	[136]
Rat BM	Rat	Alkali burn	Corneal surface	Denuded HAM	Before transplantation: BM-MSCs pre-treated with KGF-2 and autologous serum cultivated on HAM. After transplantation: ↓ corneal neovascularization, ↑ transparency, Epithelium reconstructed, Presence of K19 ⁺ epithelial cells in BM-MSCs transplanted corneas (not detected in controls)	[151]
Rat BM	Rat	Alkali-burn	Sub-conjunctival injection	-	Regeneration of corneal epithelium, ↑ neovascularization, BM-MSCs not infiltrated to the wounded cornea and remained in the injection site, ↓ infiltration of CD68 ⁺ immune cells, ↓ MIP-1α, ↓ TNF-α, and ↓ VEGF	[152]
Rat BM Cells and CM	Rat	Chemical burn	Corneal surface	-	↓ Corneal neovascularization, ↓ opacity, ↓ epithelial defect, ↓ infiltration of inflammatory and goblet cells, ↓	[153]

Source of MSCs	Host Animal	Disease Model	Route of Administration	Carrier/Scaffold	Brief Results and Proposed Reconstructing Mechanisms	Ref
Rabbit BM	Rabbit	Alkali-burn	Corneal surface	Nanofiber (poly lactic acid) Cyclosporine A	CD4+ cells infiltration, ↓ IL-2, ↓ IFN- γ , ↑ IL-10, ↑ IL-6, ↑ TGF- β 1, ↑ TSP-1, ↓ MMP-2 L- ↓ Corneal opacity, ↓ neovascularization, ↓ scar formation, restoration of corneal + thickness, ↓ MMP-1, ↓ iNOS, ↓ IL-6, ↓ α -SMA, ↓ TGF- β , ↓ VEGF	[138]
Rabbit BM	Rabbit	Alkali Burn	Corneal Surface	Nanofiber (poly lactic acid)	L- ↓ Corneal opacity, ↓ neovascularization, restoration of corneal thickness, ↓ lipid peroxidation and oxidative markers (↓ malondialdehyde and ↓ nitrotyrosine), ↓ MMP9, ↓ VEGF, ↓ u-PA, ↓ eNOS and ↓ pro-inflammatory cytokines (↓ IL-8, ↓ IL-1 β , 4 IL-2, and ↓ IFN- γ), ↑ anti-oxidant ALDH3A1, ↓ numbers of apoptotic cells	[126]
Rabbit BM	Rabbit	Alkali Burn	Intravenous	-	Infused BM-MSCs engrafted into the injured cornea and expressed α -SMA, ↑ expression of p63 and PCNA in basal epithelial cells and vimentin in stromal cells, ↓ Corneal opacity, ↓ neovascularization,	[125]
Rabbit BM	Rabbit	Alkali burn	Corneal Surface	Nanofiber (poly L- lactic acid)	Maintain corneal thickness, ↓ neovascularization, ↓ corneal opacity, ↑ re-epithelialization (↑ K3/12 expression), ↓ CD3, iNOS and caspase-3 positive cells, ↓ VEGF	[129]
Rabbit BM	Rabbit	Alkali burn	Intravenous	-	The BM-MSCs infiltrated into the injured cornea, Cornea histologic structure reconstructed	[154]
Rabbit BM	Rabbit	Chemical plus mechanical injury	Corneal surface	HAM	↑ K3/12, ↑ ABCG-2, ↑ β ₁ -integrin, ↑ connexin 43, ↓ goblet cells	[155]
Rabbit BM	Rabbit	Alkali burn	Corneal surface	Fibrin gel	Corneal surface regenerated	[156]
Human BM & Human limbal tissue	Rabbit	Alkali burn	Sub-conjunctival injection	-	↑ Healing, ↓ neovascularization, ↓ goblet cells, ↓ Opacity	[157]
Human lipoaspirate tissue	Rabbit	Alkali burn	Corneal surface	Scleral contact lens	↓ corneal opacity, 4↓ neovascularization, ↓ epithelial defect area, ↓ immune cell infiltration, ↓ fibroblastic reaction, ↓ symblepharon formation, Well organization of stroma and collagen fibers	[130]
Human lipoaspirate tissue	Rabbit	Alkali-burn	Sub-conjunctival injection	-	↑ Epithelial layers, ↓ haziness, ↑ Connexin 43, ↑ β -catenin	[158]
Human orbital fat	Mouse	Alkali burn	Corneal surface (eye-drop) and/or intra-limbal injection	-	↓ Corneal edema, ↓ opacity, ↓ immune cell infiltration, ↓ iNOS, ↑ epithelial regeneration	[159]
Rabbit inguinal fat	Rabbit	Alkali burn	Intra-stromal and sub-conjunctival injection plus corneal surface (eye-drop)	-	↓ Neovascularization, ↓ corneal opacity, ↑ re-epithelialization. Improvement in intraocular pressure, Schirmer test results and corneal histological structure, ↓ SGP, ↓ VEGF, ↑ α -SMA, ↑ Ki-67	[160]
Human limbal tissue	Mouse	Limbus to limbus epithelial debridement (LSCD model)	Corneal surface (The condition media (CM) of extracted cells were administrated as eyedrop)	-	↓ Conjunctivalization, ↓ K8-positive conjunctival goblet cells, ↑ K12-positive corneal epithelial cells	[76]

Source of MSCs	Host Animal	Disease Model	Route of Administration	Carrier/Scaffold	Brief Results and Proposed Reconstructing Mechanisms	Ref
Rat limbal tissue	Rat	Alkali burn	Eye-drop and sub-conjunctival injection	-	↓ Corneal opacity, ↓ Neovascularization, ↓ fluorescein staining score, ↓ vascular cell proliferation, ↓ vasodilation, ↓ immune cell infiltration	[132]
HAM	Rabbit	Alkali burn	Corneal surface HAM	HAM	↓ Corneal neovascularization, ↓ opacity, ↓ immune cell infiltration, improved corneal thickness, ↑ healing ratio, ↓ TNF-α	[56]
Rat Omentum	Rat	Alkali burn	Sub-conjunctival injection	-	↓ Corneal neovascularization, ↓ opacity, ↓ new vessel formation and leukocyte infiltration in the corneal stroma	[131]

Table 2: Overview of published strategies for repopulation of LESC*s* and revitalization of the limbal niche.*

	In vitro studies	In vivo pre-clinical animal Studies	Clinical Studies	Currently Available in Clinic	Strength of Evidence regarding Clinical Efficacy [†]
Repopulation of Limbal Epithelial Stem Cells					
Limbal-tissue based transplantations			[43-47, 161, 162]		A
Cultivated limbal epithelial cell transplantation	[51]	[163]	[82]		B
Non-limbal epithelial cell transplantation	[55, 66, 79]	[55, 164, 165]	[68, 78]		C
Reconstruction of the Limbal Stem Cell Niche					
Bio-active Extracellular Matrix for Limbal Niche Replacement					
Human amniotic membrane (HAM)	[166]	[167]	[39]		B
Bio-active matrices from different sources	[91, 100, 101]				C
Biological Factors to Revitalize the Limbal Niche					
Autologous/allogeneic serum eye-drops (ASE)	[110]	[105]	[168]		B
Platelet-derived factors	[108, 169, 170]	[171, 172]	[102, 109]		B
Amniotic membrane-derived factors	[88, 173]	[174, 175]	[117]		C
Cytokines and Cell-derived factors	[176, 177]	[76, 113, 178]			
Cell-based therapies for Restoring the Limbal Niche					
Mesenchymal stem cells (MSCs)	[179]	[132, 138]			

* Presence of published study-design for each regenerative strategy is shown by colors. Empty spaces mean there is no published study in the literature. Representative references for each approach study-design are also cited.

[†] Proposed based on currently published studies and expert opinion (Categorized as, A (strong), B (moderate) and C (weak)).