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Critical Appraisal of *Ex Vivo* Expansion of Human Limbal Epithelial Stem Cells

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Abstract

The stem cells (SCs) of the corneal epithelium located in the limbal basal layer are the ultimate source to maintain corneal epithelial homeostasis. Like other adult tissue-specfic SCs, self renewal and fate decision of limbal SCs are regulated by a specialized *in vivo* microenvironment, termed "niche". Loss of limbal SCs or dysfunction of the limbal niche renders corneas with a unique clinical disease labeled limbal stem cell deficiency (LSCD). Besides transplantation of autologous or allogeneic limbal SCs or amniotic membrane, a new strategy of treating LSCD is to transplant a bio-engineered graft by expanding limbal SCs *ex vivo*. Herein, we conduct a critical appraisal of six protocols that have successfully been practiced in treating human patients with LSCD, and identify issues whether niche regulation has been disrupted or maintained during isolation and expansion. Consequently, we propose a future direction that may circumvent the potential pitfalls existing in these conventional protocols by preserving the interaction between limbal SCs and their native niche cells during isolation and expansion. Such an approach may one day help realize considerable promise held by adult SCs in treating a number of diseases.

Keywords

Epithelium; *ex vivo* expansion; limbal stem cell deficiency; limbus; ocular surface; reconstruction; stem cells

INTRODUCTION

Stem cells (SCs) with extensive proliferative potential and the ability to give rise to one or more differentiated cell types are common in early mammalian embryos. By adulthood, such SCs are dispersed and kept in a unique anatomic location of each self-renewing tissue where they continue to perform remarkable and relentless self renewal to replenish the SC population lost during progeny production. Although SCs hold considerable promise for the treatment of a number of diseases, the collection of sufficient numbers of adult tissue-specific SCs and the control of their fate decision are two major obstacles to overcome.

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It becomes increasingly clear that self renewal and fate decision of adult SCs are regulated by a specialized *in vivo* microenvironment, termed "niche" (reviewed in [1,2]). Regulation of SCs in their native niche is conceivably mediated by a subset of neighboring cells (including its progeny), extracellular matrix (ECM), and factors sequestered therein. Therefore, one critical step in overcoming the aforementioned obstacles is to recapitulate the *in vivo* niche *via ex vivo* expansion of SCs in an *in vitro* environment.

Using the human corneal epithelium as a model, we will critically appraise all published protocols used for *ex vivo* expansion of human limbal epithelial progenitor cells including SCs. By analyzing whether their experimental variables have recapitulated *in vivo* niche regulation, we will identify several potential pitfalls of each protocol that may diminish the potency of bio-engineered grafts suitable for an FDA-regulated clinical trial. In the end, we will provide a forward-looking view on whether a more effective protocol can be developed by focusing on the issue of niche regulation especially by maintaining the natural close contact between SCs and their *in vivo* niche cells (NCs) during isolation and subsequent *ex vivo* expansion.

THE LIMBUS AS A UNIQUE MODEL FOR STUDYING ADULT EPITHELIAL STEM CELLS AND THEIR NICHES

Among all adult epithelial tissues, the model of the corneal epithelium is most unique in having its SCs located at the basal epithelial layer of the limbus (between the cornea and the conjunctiva), while its transient amplifying cells (TACs), i.e., the immediate progeny of SC, are located in both limbal and corneal basal epithelia [3] (also reviewed in [4a]).

When compared to the differentiated corneal epithelium, the SC-containing limbal basal layer is known to have the smallest cell size [4b], positive expression of cytokeratin (CK) 19 [5] and CK15 [6], and a high proliferative potential in different cultures [7–10]. Limbal epithelial progenitor cells are more resistant to the inhibition by tumor-promoting phorbol esters [8], and are devoid of the expression of such cornea-specific differentiation markers as CK 3 [3] and CK12 [11,12] and gap junction-mediated connexin 43 [13]. Importantly, when limbal SCs are identified by label-retaining studies in rabbits [14], not all limbal basal epithelial cells are SCs, suggesting that SCs are intermixed with their transit amplifying cells (TACs) in the limbal basal layer [4a]. The notion that not all limbal basal epithelial cells are SCs is further suggested by heterogeneous expression of vimentin [5,15], p63 [16], especially its ΔNp63α isoform [17], ABCG-2 [18–20], integrin α9 [21,22], and N-cadherin [23] in cross-sections of the limbal basal epithelium. Vimentin-expressing epithelial cells in the limbal region are thought to be a transit of limbal SCs to corneal differentiation [24]. It remains unclear whether any of these proteins can be regarded as *bona fide* bio-markers for identifying limbal SCs.

The easy access of limbal SCs owing to their unique anatomic location is more advantageous than other epithelial tissues [25] such as the bulge of the epidermis [26,27], the crypt of the intestine [28], and the intraheptic biliary tree of the liver [29]. Anatomically, the limbal epithelium contains melanin pigments [30] and is highly organized to form "limbal palisades of Vogt", where the epithelial sheet folds to increase the surface area (Fig. 1) [31]. The limbal stroma is highly vascularized and innervated [32], and is a loose connective tissue containing limbal fibroblasts. Serial histological sectioning revealed a unique epithelial crypt-like structure containing smallest basal epithelial cells [33–35], suggesting that genuine limbal SCs might lie "deeper" into the limbal stroma than expected. Furthermore, the limbus has been found to have unique ultrastructural features [36] where unique extracellular matrix components such as laminin γ3, SPARC, and tenascin-C are found [24]. The unique role of human limbal stroma in serving as a SC niche is supported by

its control of epithelial plasticity in rabbits [37], and by a recent study showing transdifferentiation of hair follicle SCs into corneal epithelial-like cells [38] when tissue recombinant experiments are performed. It remains unknown how cellular and extracellular components uniquely present in the limbal stroma might constitute the limbal niche, and whether conventional methods based on trypsin/EDTA or Dispase digestion can isolate limbal SCs together with their native NCs (reviewed in [39]).

CORNEAL DISEASES WITH LIMBAL STEM CELL DEFICIENCY (LSCD)

Ultimately, limbal epithelial SCs are responsible for the homeostasis of the corneal epithelium, a rapid self-renewing tissue (for reviews see [4a,40–42]). The importance of limbal SCs in achieving this role can be appreciated by knowing what happens when they are deficient. In rabbits, we have reported that total regeneration occurs each time when a large corneal epithelial defect is created in corneas with healthy limbal SCs. However, if limbal SCs are partially [43,44] or totally [45,46] damaged, such wounding results in abnormal corneal wound healing. Limbal deficient corneas manifest conjunctival epithelial ingrowth (i.e., conjunctivalization), vascularization, chronic inflammation, and scarring, which collectively are indicators of limbal (SC) deficiency (LSCD) (reviewed in [4a,40,47]). LSCD can be found in a number of human corneal diseases cytologically defined by evidence of conjunctivalization on the corneal surface [48] (see Table 1).

Patients inflicted with LSCD often suffer from a severe loss of vision, light sensitivity, and potential bacterial infections and are thus poor candidates for conventional corneal transplantation because only short-lived corneal TACs are transplanted and conjunctivalization may still recur. Furthermore, preexisting corneal vascularization and inflammation in LSCD increase the risk of allograft rejection. As shown in Table 1, these LSCD diseases can further be subdivided into two major categories. Category I diseases are characterized by having a clear extrinsic cause that destroys the limbal SC population. Category II diseases with diverse intrinsic causes do not have such an extrinsic destructive cause but their limbal stromal niche is altered by chronic inflammation. That is why we have speculated that the effective measure in restoring the normal phenotype in corneas with LSCD should include strategies to replenish the missing limbal SCs as well as to restore the healthy state of the limbal stromal niche (reviewed in [4a]).

CORNEAL SURFACE RECONSTRUCTION BY LIMBAL STEM CELL TRANSPLANTATION AND AMNIOTIC MEMBRANE TRANSPLANTATION

Indeed the first strategy to treat corneas with unilateral LSCD is to transplant autologous limbal SCs from the fellow eye *via* a surgical procedure termed Conjunctival Limbal Autograft, first introduced by Kenyon and Tseng in 1989 [49]. Subsequently, Tsai and Tseng in 1994 [50] noted that transplantation of allogeneic limbal SCs from cadaveric donors is effective in restoring corneas with bilateral LSCD (for historical development and classification of various transplantations see reviews in [51,52]). The second strategy to treat corneas with LSCD is to transplant amniotic membrane (AM) to restore the damaged limbal stroma, first introduced by Kim and Tseng in 1995 [53]. Since then, the popularity of using amniotic membrane transplantation (AMT) for corneal and conjunctival surface reconstruction has escalated (Fig. 2). A number of clinical studies have shown that the AM-covered ocular surface rapidly heals with reduced inflammation and scarring in the stroma (reviewed in [54–58]). Transplantation of autologous or allogeneic limbal SCs and AMT have been approved by Medicare as standard surgical procedures since January 2004 in the United States of America.

During the course of clinical investigation, we and others have learned that AMT alone is sufficient to restore corneas with *partial* (i.e., less than 360° involvement) LSCD [59–61]. Furthermore, AMT is effective in promoting the success of transplanting autologous [62,63] and allogeneic [64] limbal SCs for treating *total* LSCD. These clinical data collectively indicate that restoration of the limbal stroma by AM is as important and beneficial as transplanting limbal SCs. They also suggest that AM helps expand residual or transplanted limbal SCs *in vivo*. We have thus proposed that AM is an ideal substrate to help *ex vivo* expansion of limbal SCs in culture (reviewed in [65]).

CONVENTIONAL *EX VIVO* EXPANSION PROTOCOLS OF LIMBAL SCs ON AMNIOTIC MEMBRANE

Because of the concern of removing the limbus from a healthy eye, a new surgical approach was introduced by Pellegrini *et al* in 1997 [66] to transplant *ex vivo* expanded human limbal epithelium for treating human patients with LSCD (also reviewed in [67], see Protocol 1, Table 2). Presumably because AMT can help expand human limbal SCs *in vivo*, several groups have developed a total of five protocols for expanding limbal SCs using AM as a substrate in culture also successfully to treat human patients with LSCD (Protocol 2 to 6, Table 2). Although there are other basic and pre-clinical studies dealing with *ex vivo* expansion of limbal epithelial progenitor cells, we limited our evaluation only to these six protocols because they all have successfully been used in treating human patients with LSCD.

In short, the new surgical approach starts with a small biopsy performed at the limbal region of a healthy eye. Afterwards, these six protocols differ from one another in a number of areas before engineering an epithelium with a transplantable size. These differences can grossly be categorized according to the following 4 aspects: (1) whether limbal epithelial cells are isolated from the biopsy tissue and/or rendered into single cells, (2) whether murine 3T3 fibroblasts are used as a feeder layer, (3) whether and how AM is prepared and used as a carrier, and (4) whether air-lifting is used to promote epithelial stratification (Table 2). These six protocols use similar media containing serum, EGF and cholera toxin to support growth of limbal epithelial SCs. To meet the regulatory requirements, fetal bovine serum has successfully been interchanged with the patient's own serum in Protocols 4 to 6. At the present time, no study has systemically compared all manufacturing variables used by these six protocols. Hence it remains unclear which variable is crucial for achieving effective *ex vivo* expansion of human limbal epithelial SCs. As a result, one may question which protocol can be considered the most optimal one to adopt for an FDA-regulated clinical trial.

Herein, we would like to appraise these six protocols from the viewpoint whether these manufacturing variables disrupt the native limbal niche, and if so what measures have been taken to restore what is lost regarding the niche regulation. We identify the experimental maneuver that might not pass the regulatory requirement. Furthermore, we examine whether some maneuvers may potentially be harmful to limbal epithelial SCs during isolation and expansion based on recent research results.

1. Separation of Limbal Epithelial Sheets from the Limbal Stroma

Although poorly defined, it is plausible that limbal SCs are regulated by their native niche in the normal *in vivo* environment. Therefore, the first issue one may face is whether it is necessary to separate the limbal epithelium from the underlying limbal stroma in the limbal biopsy specimen. All except for Protocols 5 and 6 separate limbal epithelial sheets from the underlying stroma, and/or render them into single cells by enzymatic digestion. Protocols 1 and 2 use trypsin/EDTA. Protocol 3 subjects the limbal biopsy sample to a brief digestion

with Dispase but does not remove the epithelium from the limbal stroma. It has been reported that Dispase digestion results in the isolation of intact and viable human limbal epithelial sheets [68]. Protocol 4 uses Dispase digestion followed by a brief treatment of trypsin/EDTA to yield single cells. Our recent study showed that the proliferative capacity, clonogenicity, and p63-positive progenitors are better preserved in such Dispase-isolated sheets than in single cells obtained by subsequent Trypsin/EDTA treatment [69]. Therefore, human limbal epithelial sheets may lose their proliferative capacity and increase their tendency to differentiate when their intercellular junctions are disrupted by Trypsin/EDTA into single cells. As a result, one may question the validity of rendering epithelial sheets into single cells used in Protocols 1, 2 and 4. Although epithelial sheets isolated by Dispase preserves a higher p63-enriched proliferative capacity [69], it remains unknown whether Dispase removes the entire limbal epithelial SCs, niche cells or both. This concern is raised because limbal SCs lie deeper in the stroma than expected [33–35]. Assuming that limbal SCs are indeed isolated, it is still not clear whether NCs in the stroma are also included during Dispase isolation. Even if we assume Dispase-isolated human limbal epithelial sheets contain both SCs and NCs, it remains unknown whether they can be better used for *ex vivo* expansion in the future without being rendered into single cells.

Although physical contact between limbal SCs and NCs is not disrupted in Protocol 3, in which a brief Dispase digestion is used, and in Protocols 5 and 6, in which biopsy samples are mechanically minced into smaller pieces without removing the remaining limbal stroma, one major concern for these protocols is whether these limbal SCs opt to migrate out from the limbal explants onto the substrate. We have noted that limbal basal epithelial cells can also undergo intrastromal invasion when epithelial sheets are not separated from the underlying limbal stroma [70]. As a result, the growth potential and clonogenicity of the epithelial progenitor cells on the substrate decline over time [70]. This new finding raises a serious concern in these protocols that there might be a gradual loss of limbal SCs, and justifies a continued pursuit of a better method of separating limbal epithelial SCs from the underlying stroma.

2. Co-culturing with Feeder Layers

In as much as it remains uncertain whether the success of the aforementioned isolation might be hampered by disruption of intercellular interaction/support between SCs and NCs, evidence suggests that restoration of such support is crucial for *ex vivo* expansion of limbal epithelial SCs. In fact, many types of adult somatic SCs have a limited proliferative capacity when detached from their *in vivo* niche. To circumvent this problem, one common approach to *ex vivo* expansion resorts to co-culturing on a feeder layer made primarily of growth-arrest mesenchymal cells as a surrogate niche. For many types of epithelial progenitor cells, *ex vivo* expansion resorts to co-culturing on β-irradiated or mitomycin C-treated murine 3T3 fibroblast feeder layers, a technique first pioneered by Rheinwald and Green in 1975 [71]. Thus, Protocols 1 and 2 immediately seed isolated single cells on murine mitotic-arrested 3T3 fibroblast feeder layers. Although the exact mechanism remains to be elusive, it is generally believed that epithelial-mesenchymal interaction from the use of feeder layers restores the clonogenicity of epithelial progenitor cells. Therefore, one might suspect that feeder layers function like surrogate NCs in Protocols 1 and 2.

In contrast to the direct contact with 3T3 fibroblast feeder layers used in Protocols 1 and 2, Protocols 4 and 6 seed either single cells or small pieces of limbal explants on epithelially-denuded AM and then co-culture them with 3T3 fibroblasts, which are seeded on the plastic surface without a direct contact with human limbal epithelial cells. Even without a direct contact, co-cultured 3T3 fibroblasts are still effective in delaying epithelial differentiation by limbal epithelial cells seeded on denuded AM [72]. A duplex of 3T3 fibroblast feeder layers has been used to promote CK15-expressing corneal epithelial cells [73]. These results

suggest that soluble factors derived from 3T3 fibroblasts might be involved in promoting niche regulation of limbal SCs.

Even if we assume that murine 3T3 fibroblasts may serve as surrogate NCs, its use for engineering surgical graft for human transplantation does post a great regulatory concern. The FDA has issued strict guideline against the use of xenogenic cells for fear of transmitting as yet unknown murine diseases to humans.

Even if single progenitor cells can potentially be “revived” by immediate seeding with 3T3 fibroblast feeder layers, the importance of controlling the time lapsed between isolation of single cells and subsequently co-culturing cannot be overlooked. This concern is particularly relevant when single cells obtained by treatment with trypsin/EDTA are used to enrich SC population by fluorescent-activated cell sorting (FACS). One technique of FACS uses the unique property of effluxing Hoechst 33342 dye [74,75] to isolate the side population (SP) of adult somatic SCs, which preferentially express Bcrp1/ABCG2, a member of ATP-binding cassette transporters (also reviewed in [76]). Using this method, SP cells have been isolated from human [18–20], rat [77], and rabbit [20,78,79] limbal tissues. However, SP cells generate fewer clones than non-SP cells when freshly isolated limbal epithelial cells of both human [20] and rabbit [20,78,79] are cultured on 3T3 fibroblast feeder layers. One may wonder if prolonged separation from the native niche during manipulation by FACS severely hinders the clonogenicity of limbal epithelial progenitor cells.

3. Use of AM as a Substrate

Except for Protocol 1, in which fibrin gel is used as a substrate, all other protocols use either intact AM (iAM in Protocol 3) or epithelially-denuded AM (dAM) following treatment by EDTA (Protocol 4), NH_4OH (Protocol 5) or trypsin/EDTA (Protocols 2 and 4). The rationale for using either iAM or dAM is not clearly stated when they were first practiced. We have conducted a series of experiments to compare the efficacy between iAM and dAM, and our engraftment studies in nude mice have disclosed that iAM, but not dAM, preserves a limbal epithelial phenotype after *ex vivo* expansion [80]. Others have subsequently substantiated the notion that iAM can, but dAM cannot, preserve the status of limbal epithelial progenitor cells regarding the maintenance of slow-cycling properties, the lack of connexin-mediated intercellular junction, and the positive expression of p63 and CK19 [81–83]. These data collectively support the necessity of including 3T3 fibroblasts as a feeder layer when dAM is used as a substrate in Protocols 4 and 6.

To circumvent the regulatory concern of using murine cells as feeder layers, we discovered that feeder layers made of mitomycin C-arrested human amniotic epithelial cells are superior to those made of 3T3 fibroblasts in supporting expansion of human limbal epithelial SCs according to the expression of putative SC markers and the promotion of clonal growth [84]. Intriguingly, limbal epithelia cells supported by feeder layers made of human amniotic epithelial cells also exhibit plasticity in adopting neural differentiation [84]. Recently, human mesenchymal stem cell-derived feeder layers have been found to promote expression of CK3, CK15, p63α, and ABCG2 of cultured human limbal epithelial cells [85]. These results collectively explain why iAM used in Protocol 3 can circumvent the need of using 3T3 fibroblast feeder layers because limbal epithelial cells migrating from the explant are immediately in contact with devitalized human amniotic epithelial cells retained in iAM. Furthermore, if a surrogate feeder layer has to be included for *ex vivo* expansion of human limbal SCs, it may be substituted by the aforementioned human equivalents in the future.

4. Promotion of Epithelial Stratification by Air-Lifting

If, however, the limbal epithelium is not separated from the underlying stroma as shown in Protocols 3, 5 and 6, another potential drawback is the gradual loss of the limbal SC population because invading limbal basal epithelial cells also undergo epithelial-mesenchymal transition into fibroblasts in the limbal stroma [86,87]. Interestingly, using rabbit limbal explants, we have noted that air-lifting, i.e., exposing epithelial cultures to the air-medium interface, further promotes intrastromal invasion and epithelial mesenchymal invasion by limbal basal epithelial progenitor cells [86]. This experimental maneuver of air-lifting is known to promote epithelial stratification [37,86,88]. However, we recently reported that stratification promoted by air-lifting is coupled with squamous metaplasia in human limbal explants [88], which may reduce the efficacy of being used as a graft. Therefore, it remains to be determined whether airlifting used by Protocols 4 and 6 to promoted epithelial stratification is necessary for engineering limbal epithelial sheets before human transplantation.

EXPANSION PROTOCOL BY PRESERVING PHYSICAL CONTACT BETWEEN LIMBAL SCs AND NCs

The aforementioned appraisal let us conclude that there are potential pitfalls in each of the six protocols currently used for manufacturing human limbal epithelial graft for human transplantation. For Protocols 1, 2 and 4, in which human limbal epithelial sheets are isolated from the limbal stroma and then rendered into single cells, the success of *ex vivo* expansion relies on the verification that limbal SCs are indeed removed from the limbal stroma, and whether their progenitor status is successfully maintained by surrogate 3T3 fibroblasts. As stated above, meeting FDA requirements is difficult with the use of murine 3T3 fibroblasts. Future studies are needed to see if human equivalent feeder layers based on human amniotic epithelial cells or human mesenchymal stem cells can be a more effective substitute. For Protocols 3, 5 and 6, in which human limbal epithelial cells are left in contact with the underlying limbal stroma, the success of *ex vivo* expansion relies on the verification that limbal SCs indeed migrate out of the explant, and if so whether the progenitor status of migrating limbal epithelial cells is preserved by devitalized amniotic epithelial cells (Protocol 3) or by 3T3 fibroblast feeder layers (Protocol 6).

Taken together, we believe that the efficacy of the above 6 reported *ex vivo* expansion protocols can be improved by addressing the issues raised above. We suspect that the native limbal niche environment is more supportive of SC expansion than surrogate feeder layers. Therefore, it is our belief that *ex vivo* expansion of limbal SCs can further be optimized by developing a protocol focusing on the preservation of the close contact between limbal SCs and their NCs during isolation and expansion. The development of such a protocol relies on the realization that limbal SCs are closely associated with their NCs in the limbal niche, and the success of identifying and isolating NCs. To prove the hypothesis that the close contact between limbal SCs and their native NCs is more supportive than that with surrogated feeder layers, one will first require the successful identification and isolation of limbal NCs. Furthermore, a new protocol can be devised by preserving the close contact between limbal SCs and their native NCs during isolation and expansion. Such a protocol may meet the requirements for initiating an FDA-approved Phase I clinical trial so as to determine the safety and efficacy of this new bio-engineered corneal surface tissue in treating patients inflicted with total LSCD.

PROPRIETARY INTERESTS

SCGT and S-Y Chen, but not others, have filed a patent on the method and clinical uses of *ex vivo* expansion of epithelial progenitor cells.

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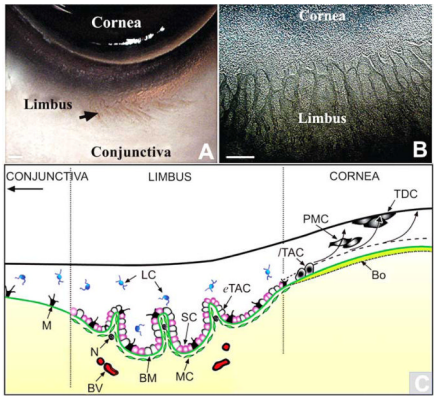

Fig. (1). The Limbal Palisades of Vogt

Palisades of Vogt (arrow) are readily recognized in the human limbus (**A**). Such a unique pigmented structure can be identified on the flat mount preparation of dispase-isolated human limbal epithelial sheets (**B**, Bar represents 500 μm in **A** and **B**). Schematic drawing of the limbal epithelium and the limbal niche shows the cellular components of SC, TAC, PMC (post-mitotic cells), TDC (terminally differentiated cells), M (melanocytes), LC (Langerhan's cells) and MC (presumed NC) as well as BM (basement membrane), Bo (Bowman's layer), N (nerves) and BV (blood vessels) (both modified from [39] with permission).

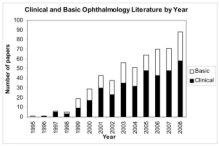

Fig. (2). Clinical and Basic Ophthalmology Literature Regarding to AMT by Year

Over the past decade, there has been a surge of interest in amniotic membrane transplantation (AMT) for ocular surface reconstruction. PubMed search of papers published in Ophthalmology per year are plotted from the PI's pioneering paper in 1995 [53]). These studies collectively showed that AMT is effective in facilitating epithelial wound healing and reducing stromal inflammation, scarring and unwanted new blood vessel formation.

Table 1

Human Corneal Diseases Characterized by Limbal Deficiency

I. Destructive Loss of Limbal Stem Cell Population

- Chemical or Thermal Injuries
- Stevens-Johnson Syndrome or Toxic epidermal necrolysis
- Multiple Surgeries or Cryotherapies to the Limbus (Iatrogenic)
- 5-fluororacil toxicity
- Contact Lens-induced Keratopathy
- Severe Microbial Infection

II. Dysfunction of Limbal Stromal Microenvironment

- Aniridia (hereditary)
- Keratitis Associated with Multiple Endocrine Deficiency
- Neurotrophic Keratopathy (Neuronal or Ischemic)
- Radiation-induced keratopathy
- Mustard Gas-induced keratopathy
- Peripheral Corneal or Limbal Inflammation and Ulceration
- Pterygium and Pseudopterygium
- Idiopathic

Table 2

Ex Vivo Expansion Protocols of Autologous Human Limbal Epithelial Cells Practiced Successfully in Human Patients

Protocols		1 Pellegrini 1997 [66]	2 Schwab 2000 [89]	3 Tsai 2000 [90]	4 Nakamura 2004 [91] and 2006 [92]	5 Sangwan 2003 [93]	6 Shimazaki 2002 [94] and Kawashima 2007 [95]
Epithelial Isolation from Limbal Biopsy		Trypsin/EDTA	Trypsin/EDTA	Brief Dispase	Cut into small pieces or Dispase and Trypsin/ EDTA	Cut into Small pieces	Cut into Small pieces
3T3 Coculturing for Pre-amplification		Yes	Yes	No	No	No	No
Substrate		Fibrin	dAM by Trypsin/EDTA	iAM	dAM by EDTA and scraping	dAM by Trypsin/EDTA and scraping	dAM by NH_4OH and scraping
Medium	**Base Medium**		D or K	D/F(1:1)	K	M/F(1:1)	D/F(1:1)
	Serum		10% FBS then serum-free	5% FBS	5% FBS or HS	10% FBS, or HS	5% FBS or HS
	EGF	+	+	+	NA	+	+
	Hc	+	+	+	NA	+	–
	ITS		–	+	NA	+	–
	CTX		+	+	NA	+	+
	DMSO		–	+	NA	–	–
Co-culturing with 3T3 on Plastic		No	No	No	Yes	No	Yes
Air-lifting		No	No	No	Yes	No	Yes
Days to Achieve a Transplantable Size		14–21	NA	14 – 21	at least 14	14 – 21	14

[Not]: Abbreviation used: CTX: Cholera Toxin; D: DMEM; D/F: DMEM/F12; dAM: denuded AM; EGF: Epithelial Growth Factor; FBS: fetal bovine serum; Hc: Hydrocortisone; HS: Human Serum; iAM: intact AM; ITS: Insulin Transferrin Selenium; K: KGM; M: MEM.