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## Review Article

# A Growing Research Area of *In vitro* Cell Culture Technologies for Corneal Surface Regeneration: A Short Communication of Basic Steps to Cultivate Limbal Epithelial Stem Cells -

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## INTRODUCTION

### Limbal stem cell niche and limbal stem cell deficiency

Cornea is the outermost anterior layer of the eye ball which has unique optical and physio-mechanical properties to maintain good vision. A stratified squamous epithelium is continued with the conjunctival epithelium in the limbal area and create a protective stratum on the ocular surface to defend internal structure from environmental threats [1]. The dynamic equilibrium of the epithelial turnover is maintained by limbal niche where the Limbal Epithelial Stem Cells (LESCs) reside in an excellent arrangement. Palisades of Vogt form a sheltered region for LESCs to sustain their self-renewing capacity and act as a chief factory in order to preserve stem cell pool and proceed centripetal stem cell migration and differentiation into central corneal epithelial cells [2]. Various ocular or systemic disorders can disturb limbal area and cause decrease or loss of LESCs which is so-called Limbal Stem Cell Deficiency (LSCD). Clinically, it is diagnosed by the presence of conjunctival cells and vessels on the cornea. Because of the demolition of the LESCs' barrier function in the limbal region, conjunctival cells can easily migrate through corneal surface and distort the optical clarity of the cornea. The symptoms including photophobia, epiphora, blepharospasm, reduced visual acuity, and severe pain resulted in extremely severe discomfort of the patient which is subsequently lead to decrease in the quality of life. To ensure the diagnosis, corneal impression cytology can be performed to visualize goblet cells in the specimen [3].

### Treatment strategies

After definite diagnosis, treatment modalities come into question which depend on the severity of LSCD. To date, assorted methods have been established which have diverse success rates for both clinical and pre-clinical purposes. Initially, small-to-large pieces of limbal tissue (explant) obtained from auto- or allo-origin was transplanted to the diseased eye by Kenyon and Tseng in 1980s [4]. However, the major drawbacks such as requirement of large free grafts and possible induction of iatrogenic donor-site deficiency in the healthy eye for autograft procedure, and limited availability of qualified donor graft material and possible tissue rejection for allograft procedure cause the development of novel treatment strategies.

Together with the improvement in the field of cell and tissue culture, treatment modalities oriented toward cell therapies which in fact based on regenerative medicine. The initial report of Cultivated Limbal Epithelial Transplantation (CLET) by Pellegrini et al. [5] inspired investigators to explore different cell sources and cultivation methods to regenerate corneal surface [6]. Both established and developing techniques have been shown to be successful, however the question of which technique is the most innocuous for clinical purposes remains to be answered. Furthermore, a recent modification of limbal tissue transplantation; Simple Limbal Epithelial Transplantation (SLET) which actually is an *in vivo* epithelial co-culture of corneal and conjunctival cells on Human-Derived Amniotic Membrane (HAM) has gained popularity by some clinicians because of its ease of application and low cost [7].

### Basic principles of *in vitro* limbal epithelial stem cell cultivation

**Tissue and cell sources:** In order to regenerate corneal surface various tissue and cell sources have been demonstrated such as cadaver or living donor (allograft) limbal tissue, patient's healthy eye limbal tissue (autograft), oral mucosal cells, nasal mucosal cells,

Mesenchymal Stem Cells (MSCs), Induced Pluripotent Stem Cells (iPSCs), and cell lines obtained from cell banks [6,8-12]. Although this highly wealthy cell and tissue source, limbal explant tissue obtained from an auto- or allo-origin are generally the most popular preferences for researchers because of their proximity to natural limbal niche. Oral mucosal cells are the second most preferred cell source not only for corneal surface but also conjunctival and whole ocular surface regeneration [11]. More recently, bone marrow- or adipose-derived MSCs have increasingly gained popularity because of their high capacity to differentiate epithelial, stromal or endothelial cells which are three main cell types of corneal layers [9,13]. Another cell source is a reprogrammed stem cell, called iPSC, that has been first discovered in 2006 at Kyoto University. Yamanaka et al. identified some alternative methods that would provide specialized adult cells to be reprogrammed to an embryonic stem cell-like state. Since this initial discovery, investigators opened up exciting possibilities to treat various ocular diseases such as age related macular degeneration, Stargardt disease, and LSCD [10]. The similarity between embryonic stem cells and iPSCs means that this kind of cells can be used to derive any kind of cell type in the human body under precisely controlled cell culture conditions and may also provide an unlimited supply of regeneration capacity for many patients with untreatable diseases.

**Isolation of LESCs via limbal explant & enzymatic digestion methods:** After the obtainment of limbal tissue (explant) from the other eye, cadaver, or a living donor the specimen transferred to laboratory in Optisol™ solution under sterile conditions. There are two ways to isolate desired cells (LESCs): (i) enzymatic digestion method, (ii) limbal explant cultivation. The former consists of a serial of enzymatic treatment including collagenase and trypsin to separate epithelial stem cells from basal membrane and adjacent cells such as melanocytes, stromal cells, and dendritic cells [14,15]. Following this, the cell suspension transferred to culture surface and wait for the individual cells to attach and proliferate on the substratum. However, because of the disruption of the niche structure and leave the cells alone without the support of a real limbal mechanical support, the cells can be slugged to exhibit stem cell characteristics. The latter does not require any enzymatic transaction in which the limbal tissue specimen directly transferred to culture surface to obtain migrating cells that are supposed to be LESCs [15,16]. Nevertheless, it is important to provide *in vivo* niche standards to induce stem cell migration towards the culture substratum with appropriate surface treatments and medium formulations. There have been several culture conditions consisting of defined medium formulations to differentiate various cell types into corneal epithelial progenitor cells [17]. The debate on which technique is the better way to achieve desired success has still been proceeded among researchers, therefore investigators can choose the cultivation method based on their laboratory experience.

**Culture conditions (substratum & medium):** Another hot point is the scheme of the cell culture conditions including cell culture substratum and medium. The initial green medium consisting of Dulbecco's Modified Eagle Medium (DMEM)/F12 with fetal bovine serum, cholera toxin, insulin, hydrocortisone, L-glutamine, and antibiotics was determined in 1975 [18]. However, the ingredients exhibit non-optimal level of biosafety in terms of their production process and origin such as bacterial cultures on bovine brain broth and fetal calf serum [19-21]. Therefore, the risk of micro-chimerism, immune rejections, and transmission of pathogens are still existed that are really harmful for individual's life. Similarly, the culture surface modifications with murine NIH 3T3 feeder layer, HAM,

and commercially available collagen or fibrin matrices are known to pose the threat of transmission of animal-derived pathogens, donor tissue-derived infections, and even high cost [22,23]. Recently, the attempt was made by some researchers to minimize the amount of biohazardous ingredient in culture formulations that is called xenobiotic-free cell culture systems. To this end, feeder free conditions and human-derived carriers, feeder cells, and serum have been reported with various success rates [19-21,24,25]. Additionally, hyaluronan hydrogel scaffold-based xeno-free culture system that provided ready-to-use cell monolayer was proposed to maintain similar characteristics of native corneal epithelium [26]. Because the main goal is to achieve nearly the same success rates with the conventional methods, investigators are still try to introduce the safer and applicable cell culture system.

**Cell sheet technology:** Cell Sheet Technology (CST) is a bottom-up approach of tissue engineering in which nanotechnological cell culture surface treatment is applied [27]. During the 2000s, Yamato et al. discovered a novel surface modification method using a temperature-responsive polymer named Poly (N-Isopropylacrylamide) (PNIPAM) to obtain an intact cell sheet at the end of the cell culture procedure [28]. In this technology, the PNIPAM polymer is covalently grafted onto the cell culture dish surface with a thickness of 20-30 nm that allows to control of cell adhesion and detachment because of its shifting hydrophobic-hydrophilic feature in a temperature-responsive way [29]. The surface is relatively hydrophobic at 37°C but turns hydrophilic under Lower Critical Solution Temperature (LCST) which is generally about 32°C. Similar to conventional cell culture methods, epithelial cells adhere, spread, and proliferate on the cell culture dish surface at hydrophobic state (37°C), then the temperature is reduced below LCST (32°C) that allows the polymer chains to lengthen and push the contiguous cell sheet without detriment to cell-cell junctions and Extracellular Matrix (ECM) [30]. Because of the lack of any proteolytic enzymatic interaction, cells can keep their surface characteristics, especially receptor and junction proteins, therefore a functional small tissue construct can be obtained with cells that have native functions [28]. By this technology, the use of carrier and feeder layer is excluded which facilitates to create a biologically safe cell culture condition. The cell sheet includes deposited ECM that enables direct transplantation of cell sheets to host tissues without fixative or sutures that increases patient's comfort during and after the surgery [31]. Moreover, a multi-layered cell sheet can be obtained by automated cell culture system to mimic the corneal real structure *in vivo* [32].

**Characterization:** Before the clinical applications, harvested cell sheets have to be characterized with various methods such as morphological imaging with Phase Contrast Microscopy (PCM) or Scanning Electron Microscopy (SEM), putative marker expression with immunohistochemical staining or flow cytometry, and gene expression with Polymerase Chain Reaction (PCR). During the cell culture studies, the proliferating or outgrowth cells can be visualized by live cell imaging systems (Juli™) or PCM. The small cells that have round to cobblestone-like morphology and high nucleus-to-cytoplasm ratio refer to LESC-like state that can easily discriminated from fibroblastic cells. However, advanced staining procedures like skeleton/nucleus staining and live/dead staining can be performed to investigate the morphology and viability of harvested cells. Although the morphological imaging shows us a lot of cue to suppose the cell origin, protein and gene expression analysis should be performed for definite characterization. For this reason, to prove stemness of

the cells putative stem cell markers such as tumor protein 63, ATP-binding cassette protein2, and integrin  $\alpha 9\beta 1$  used for the analysis. Differentiated epithelial cell markers (K3, connexin 43, K12) may also be used for the discrimination of central corneal epithelial cells from LSCs [33,34]. Apart from the cell stemness, it is also very important to show cellular interactions over specific membrane proteins to prove the functionality of the acquired cell sheet. Investigators should perform all the analysis in accordance with the manufacturer's recommendations to rule-out incorrect results.

**Transplantation:** The last and the most important step is to safely transplant the harvested cell sheet to host tissue. The HAM is the most preferred carrier that serve a basal membrane function for cells to grow, migrate and proliferate easily on it. Additionally, a continuous cell sheet constructed onto a HAM can be transferred to surgery room under sterile conditions that allows surgeons to fix the cell sheet onto the cornea with fixative glues or sutures. However, a more recent technology, CST, offers superior proposal that does not required additional surgical material to secure the cell sheet. Because of the shortcomings of the HAM such as biological instability, lack of optimized preparation protocols, and risk of contamination and transmission of infection, CST is a conceivable procedure to cultivate and transplant the LSCs [31].

## CONCLUSION

*In vitro* LESC culture in the treatment of LSCD is a recent development in the field of tissue engineering and regenerative medicine. After the initial report by Pellegrini et al, too many investigations have been published for the best pre-clinical and clinical success, however the technique of LESC expansion is in its infancy. In the years to come, with the improvement of understanding of limbal niche and LESC characteristics the best way to obtain high success rates will be emerged. Consequently, the importance of the cell therapies should be elaborately considered and the effort for the discovery of new definite cell culture methods should be spent which will provide to prevent the development of LSCD in the first place.

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