Javid D, Zhang J, Alamdari DH. Hot Topics in Limbal Stem Cells Culture: Mini Review.
J Ophthalmol Sci. 2021;3(1):1-5

Journal of Ophthalmological Science

Mini Review Article Open Access

Hot Topics in Limbal Stem Cells Culture: Mini Review

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Article Info

Article Notes
Received: November 16, 2020
Accepted: January 06, 2021

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Keywords:
Limbal stem cells
CLET
Substrate

Abstract

One of the most effective treatments for Limbal Stem Cells Deficiency (LSCD) is Cultivated Limbal Epithelial Stem Cells (CLET). Precise knowledge of limbal stem cells culture increase the quality of treatment, mitigate the side effects and complications of organ transplantation, and expedite the treatment process. However, protocols for CLET need further optimization and standardization. This mini review covers basic concepts and important criteria that should be taken into consideration in designing a successful CLET that can be helpful for other targets of regenerative medicine.

Limbal Epithelial Stem Cells (LESCs) refer to unipotent adult stem cells (ASCS) capable of extensive self-regeneration. LESCs play anti-angiogenic and anti-inflammatory roles in fostering the integrity of the whole corneal surface and inhabiting at limbus. Limbus is made of a profound and complicated network of crypts enclosed by the palisades of Vogt, which creates a niche for corneal epithelial stem cells. During the normal corneal epithelial performance, LESCs stay in the limbus where they are split asymmetrically, forming the first generation of novel basal corneal epithelial cells – transient amplifying cells (TACs) in the peripheral cornea. They are more activated over episodes of considerable wound healing where the corneal surface regeneration comprises division, migration, and maturation of these cells[1]. However, it seems that the shape and dimensions of a corneal epithelial injury determine the involvement of LSCs in the wound healing process as it has been shown in a mouse study that smaller circular wounds with 0.75-mm diameter do not rely on LSCs as do larger wounds[2]. Given their engagement in the regeneration of healthy epithelium, LESCs are utilized for the treatment of corneal epithelium degeneration induced by external or genetic factors.

Limbal stem cells markers could be characterized using markers or mechanical properties. Negative markers includes molecules expressed in differentiated epithelium (cytokeratins (CK3, CK12), conjunctival epithelium (CK5, CK14, CK19), transitional cells (Vimentin), transient amplifying cells (α9 and β1 integrins, α-enolase, and connexin-43)[3]. Positive markers include ΔNp63α, C/EBPσ, Bmi1, ATP-binding cassette transporter (ABCG2), ATP-binding cassette, sub-family B, member 5 (ABCB5) and Notch-1. The nuclear transcription factor of ΔNp63α, is a major factor affecting the proliferative capacity of stem cells in stratified epithelia. In cultures where more than 3% are accounted for by p63, the rate of transplantation rate success is near 80%[4]. Both C/EBPσ and Bmi1

are believed to regulate LSC senescence, and the expression of them is downregulated in response to corneal injury[5]. Research suggests that ABCG2+ cells are not linked to elevated colony-creating efficiency[6]. Notch-1 has been recognized in a subclass of ABCG2-expressing cells[7]. ABCB5 is required for corneal development, LESC homeostasis and regeneration[8]. Recently, mechanical properties as phenotypic and label-free markers such as cell stiffness and size have been in the spotlight as stemness indicator in the corneal limbus. It has been proved that LSCs have small diameter and high nucleus/cytoplasm ratio in comparison with other cells from the limbus. In addition, they are larger than central cornea cells but smaller than differentiated limbal epithelial cells (LECs) and considerably softer than both the central cornea cells and the differentiated LECs[9]. Because using antibodies to identify LSCs *in vitro* is inconvenient, time consuming and cellular physiology can be subsequently affected, label-free cell-enrichment approaches alongside antibody-based methods can greatly develop LSCD treatment by enabling a faster and cheaper process to identify stem-like cells.

Limbal Stem Cell Deficiency (LSCD) is a condition arising from the inadequate quantity and/or dysfunction of LSCs. It is characterized by conjunctivalization and inflammation of corneal surface and neovascularization of corneal stroma. This may result in pain, low vision, photophobia, and finally corneal blindness. There are different categories for LSCD etiology: primary and secondary causes, acquired immune-related, non-immune and hereditary causes, and more unfavorable and favorable. Unilateral LSCD is primarily caused by chemical injury, which accounts for 66–75% of cases. One major cause of bilateral LSCD is Stevens–Johnson syndrome/toxic epidermal necrolysis (SJS/TEN)[10]. Unilateral LSCD is more prevalent than bilateral LSCD. It commonly affects young male subjects, with developing total LSCD[11].

Treatment of Limbal Stem Cell Deficiency (LSCD) is a function of the extent of the limbus' involvement (sectoral vs total) as well as the unilateral or bilateral nature of the disease. With regard to partial LSCD, there are a number of conservative nonsurgical alternatives, including autologous serum drops, therapeutic soft contact lens, therapeutic scleral lens, topical lubrication as well as some conservative surgical alternatives such as corneal scraping and amniotic membrane transplantation (AMT)[12]. Regarding the total ocular surface diseases, the latest treatment includes the application of cultivated corneal limbal epithelial cell transplantation. It encompasses conjunctival limbal autograft(CLAU), conjunctival limbal allograft (CLAL), Keratolimbal allograft (KLAL), simple limbal epithelial transplantation (SLET) and ex vivo cultivated limbal epithelial stem cells (CLET)[13].

Cultivated Limbal Epithelial Stem Cells (CLET) has been successful in the treatment of partial, unilateral, or total LSCD. This technique is characterized by rapid epithelialization, little inflammation, and the requirement of little donor tissue. The general success rate of this process is considered to be 70%–80%[14]. Concisely, limbal epithelial cells are harvested through a small limbal biopsy. LESCs, located in the limbus, are isolated and cultured in a laboratory to produce a sheet of cultured LECs that is suitable for transplantation onto the cornea. EMA approved the use of ex vivo-expanded autologous human corneal epithelial cells containing stem cell transplantation (Holoclar®) in 2015 as a cure for patients with moderate or severe burn-related LSCD.

Sources of tissue for cultivating limbal stem cells may be patients (autograft). Accordingly, it often comes from the healthy eye of the patient's in unilateral cases, from another individual (allograft), from a cadaver or living relative in bilateral cases or from animals (xenograft). Studies demonstrate that changes in the elasticity of human cornea have an effect on the cellular behavior of LSCs. Hence, younger donor age is associated with better outcome[15]. According to recent research, the success rate of allogeneic and autologous CLET is not significantly different in patients with LSCD[16]. The most recent advances in therapeutic methods suggest that epithelial cells could be harvested from non-ocular sites including oral mucosal epithelial cells, conjunctival epithelial cells, hair follicle bulge-derived epithelial stem cells, amniotic epithelial cells, human embryonic stem cells, induced pluripotent stem cells, umbilical cord lining epithelial stem cells and Wharton's Jelly mesenchymal stem cells, mesenchymal stem cells, immature dental pulp stem cells[17].

Methods of Limbal Stem Cells Expansion

Explant culture or cell suspension culture. In the cell suspension culture, the biopsy specimen derived from the limbal area is disassociated into separate cells by enzymes including trypsin, dispase, and collagenase, then seeded on a substrate carrier to be further transferred to the ocular surface. In the explant culture, it is customary to put the explant tissue on a substrate, which enables the growth and proliferation of cells on the substrate surface. Findings suggest that explants from the Lconj (located outermost adjacent to the conjunctiva) and Lm (middle limbus) sites should be selected for limbal cell expansion for CLET[18]. The graft size should be a minimum of 0.3 mm^2 for live explant and 0.5 mm^2 for cadaveric explant[19]. Furthermore, organ culture systems require a bio-adhesive to improve the adhesion of the cultivated cells on the carrier. Apparently, the best bio-adhesive is fibrin glue (FG), compared to xx and yy etc[4]. Cell suspensions formed from fresh tissue is more likely to provide a larger and more viable stem cell population than that obtained from organ culture–stored human limbal epithelium[20]. However, organ-culture

conditions can preserve limbal cell mitotic potential if limbal tissue is excised early after circulatory arrest[21].

2D or 3D culture techniques. Use of LSCs for clinical applications requires a high quality and quantity of cells. This requires large-scale expansion of LSCs followed by efficient and homogeneous differentiation into functional results. Traditional approaches for preservation and expansion of cells rely on two-dimensional (2-D) culturing methods using plastic culture plates and xenogeneic media. These methods provide limited expansion as a monolayer and cells tend to lose clonal and differentiation capability upon long-term passaging. To address the various problems facing 2-D culture of SCs, three-dimensional (3-D) culture methods have been developed. Natural 3-D niches allow for interactions between cells, inclines of nutrients, oxygen, and waste. Recently, new approaches for the expansion of LSCs have emphasized 3-D cell growth to mimic the *in vivo* environment, including, 3D culture system of clusters of LSCs on bone marrow-derived mesenchymal stem cells (3D-CC-BM)[22], Real Architecture For 3D Tissue and tissue equivalents (RAFT-TEs)[23], laser-assisted 3D bioprinting (3D LaBP)[24], cultivation of limbal tissue explants for more than 3 month in medium without using scaffolds[25], 3D culture system of limbal epithelial cells on limbal mesenchymal cells (3D CC-LMC)[26] and 3D culture system of limbal tissue explant on amniotic membrane using platelet-rich plasma-Fibrin glue (3D AM-PRP-FG)[27]. The 2D culture lasts for about 2 weeks, which could be extended using the air-lifting technique. In this method, the culture is subjected to the air-liquid interface to produce a multilayer epithelium[28]. However, through constructing 3D scaffold to decrease exposure to the oxygen as an air-lifting technique, the culture length can remain 2 weeks[27].

Xenogeneic or xenogeneic-free. Traditionally, LSCs often require undefined or xenogeneic materials including attachment substrates such as feeder layer of mouse 3T3 fibroblasts, culture medium containing xenogeneic cytokines and growth factors, as well as fetal bovine serum (FBS). Use of xenogeneic or animal derived media can potentially transmit pathogens, cause severe immunologic reactions in recipients and limit reproducibility between cultures due to lot-to-lot variation of the material used. Furthermore, studies have shown that the composition and organization of the extracellular matrix have important roles in cell differentiation through sending biochemical and mechanical signals[29]. The U.S. Food and Drug Administration (FDA) have imposed strict guidelines against xenobiotic-transplantations and the use of xenogeneic cells during production of tissue[30]. Subsequently, due to recent concertation on xenobiotic-free culture systems, utilization of autologous serum like human serum, substrate such as autologous FG, human-AM and autologous PRP and xenobiotic-free culture media have expanded[31]. Among all the xenobiotic-free conditions tested, modified supplemented hormonal epithelium medium (mSHEM) was the most efficient and consistent in supporting LSC phenotype and growth[32].

Diverse substrates and carriers have been presented to cultivate limbal stem cells in an attempt to improve the clinical outcomes. The long-run viability of donor LSCs could be undermined by the absence of a supporting and healthy LSC niche. The solution is adopting an optimal substrate to mimic the mechanical properties and transparency similar to that of *in vivo* which may be solid, semi-solid or liquid. LSCs niche is twice as soft as adjacent tissue of the cornea and sclera , as stem cells require very soft substrates in order to maintain stemness[33]. In addition, it has been shown that smooth surfaces with pore sizes <1 μm stimulated corneal epithelial cell migration, while rough surfaces with pore sizes >1 μm lessened migration[34] and discontinuous shear flow induced differentiation, but constant shear flow preserved the stemness of LSCs[35]. Consequently, as studies demonstrate, the phenotype of LESCs is highly dependent on the mechanical properties of their substrate[36], biochemical aspects of chosen substrate should be taken into consideration including, elastic properties, surface topography, porosity and shear flow as nanotopographic feature.

Furthermore, it is essential that the carrier system can be degraded in the body. Such carriers include: (i) cell-free biological materials including, modified human amniotic membranes, Fibrin, Natural Collagen, Chitosan-gelatin, Keratin, PRP[22], Human Processed Lipoaspirate cells (PLA)[37] Epithelial basement membrane of human decellularized cornea[38] and Descemet's membrane[39] (ii) plastics that are compatible with the body such as, plastic compression collagen, siloxane hydrogel contact lenses, poly(-caprolactone), polymethacrylate, hydroxyethylmethacrylate, and (iii) physiologically degradable synthetic polymers and biopolymers such as chemically crosslinked collagen, silk fibroin, human anterior lens capsule, poly (ethylene glycol), poly (vinyl alcohol), poly(lactide-co-glycolide)[40].

Human amniotic membrane is presently the most prevalent substrate in clinical applications of CLET, but it suffers from a number of drawbacks such as a theoretical peril of infection, biological instability, and lack of standardized preparation protocols. Therefore, lately, to build more accurate and functional replicates of native tissue structure, more synthetic scaffolds have been developed as they resemble physical and chemical properties of tissue ECM including, microfabricated scaffolds[40], electrospun scaffolds[41], synthesized carboxymethyl cellulose[42] and novel collagen scaffolds[43].

Future Perspectives and Conclusions

The potential clinical application of LESCs phenotype-through-biomechanical modulation is a new concept that has recently been considered. As transplantation success rate is strongly dependent on the percentage of LESCs within the transplanted cells, the major challenge in the improvement of transplantation techniques is the identification of the LESCs and limbal stem cells mechanobiology, which would allow enrichment of the stem cell content of the transplant. Therefore, further research is needed to build functional *in vitro* constructs or cornea substitutes for modulating tissue biomechanics or controlling stem cell phenotype and it is necessary to clarify the mechanisms important for LSC maintenance, including mechanical features and biomaterial signaling that would lead to the uniform and reproducible expansion of LSCs without loss of stemness or differentiation potential destined for the clinic.

Through optimum expansion techniques and scaffolding in tissue engineering strategies for the regeneration of cornea tissue and other tissues, advanced clinical translation of a range of stem cell types is promising for any condition that is expected to be successfully treated with stem cells. Such diseases can be, for example, graft rejection, tumors, lung, liver, kidney disease, a connective tissue disease, a cardiovascular disease, metabolic disease, neurodegenerative disease, autoimmune disease, anemia, hemophilia, diabetes, ischemia, inflammation, an infectious disease, a genetic disease defect as well as aging processes and, in general, any organ whose function is to be restored or damaged tissue whose specific cell types can be cultivated with the present method such as, nerve tissue, muscle tissue, skin, connective tissue, cartilaginous tissue, bone tissue, glandular tissue, neuromuscular tissue, gastrointestinal tissue, vessels, lung, heart, liver, kidney, blood cells, etc.

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