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Limbal stem cell deficiency: special focus on tracking limbal stem cells

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Abstract: The cornea, the transparent part of the eye that helps in the formation of images, has multiple layers. Among them, the superficial epithelial layer is protected and maintained homeostatically by the limbus, located in the periphery of the cornea. Limbal stem cells are located at the basal layer of the limbus and the loss of these cells is called limbal stem cell deficiency (LSCD). LSCD is characterized by corneal vascularization, inflammation, and conjunctivalization, which ultimately lead to blindness. Several clinical transplantation procedures used for the treatment of LSCD are cultivated limbal epithelial transplantation, simple limbal epithelial transplantation, and cultured oral mucosal epithelial transplantation. Long term follow-up of these mentioned transplantations across the globe shows that the success rate declines as the follow-up period increases. There is an urgent need to know the mechanism of stem cell transplantation in curing LSCD to improve the success rate of the transplantations. There are numerous reports on other stem cell transplantation procedures and its mechanism in healing the disease or deficiency. In the case of LSCD a lot of labeling and tracking methods are used, but there are no confined reports on the mechanisms of the stem cell transplantation and its success. This review summarizes the current treatments available for LSCD, substrates used during limbal stem cell culture and transplantation, and findings from posttransplant cell tracking experiments conducted up to date.

Key words: Limbal stem cell deficiency, limbal epithelial transplantation, limbal stem cells, stem cell transplantation, cell tracking, cornea

1. Introduction

The unique structure of the cornea, the transparent and curved surface of the eye, grants it powerful refractive capabilities that are critical for sight. A multilayered epithelium composes the outermost region of the cornea, protecting the inner corneal strata and intraocular structures against infection and injury. The cornea undergoes orderly desquamation as superficial epithelial cells are sloughed off and replaced by deeper cells with complete turnover occurring every 7 days (Bartlett and Jaanus 2008). Cells of the basal stratum, in conjunction with limbal stem cells (LSCs) located in the periphery of the cornea, play a central role in maintaining corneal epithelial homeostasis.

Current knowledge of the processes governing the homeostatic maintenance of the corneal epithelium can be summarized in the following theories: 1) corneal epithelial stem cell hypothesis (Majo et al., 2008); 2) limbal epithelial stem cell hypothesis (Lehrer et al., 1998); and 3) germinative basal layer hypothesis (Haddad and Faria-e-Sousa, 2014). According to the corneal epithelial stem cell hypothesis, stem cells scattered throughout the cornea are responsible for replenishing the epithelium during normal homeostasis, while stem cells residing within the limbus are activated only at the time of wound repair. Conversely, the limbal stem cell hypothesis states that under normal conditions, LSCs repopulate the epithelium. These cells migrate from the limbal crypt at the corneoscleral junction into the basal corneal epithelium, where they proliferate and move towards the corneal surface. In the event of corneal insult, LSCs are stimulated to proliferate more rapidly. In contrast with the previous models, the germinative basal layer hypothesis proposes that the cells of the corneal basal epithelium are responsible for the renewal of the superficial epithelium, with no significant involvement of the limbus. Of these competing theories, the limbal epithelial stem cell hypothesis has been most favored by experimental evidence (West et al., 2015).

To identify the cell source behind epithelial regeneration, early studies compared the cell replication rates of different epithelial strata within the limbus. The use of mitotic figure counts and thymidine incorporation assays revealed that the basal cell layer possesses high proliferative capacity (Buschke et al., 1943; Hanna and O'Brien, 1960). In addition, the centripetal migration of the melanin pigment cells located at the corneal periphery

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in both rabbits and humans further implies the presence of a stem cell source within the limbus (Mann, 1944; Davanger and Evensen, 1971).

The loss of limbus from various causes leads to a condition known as limbal stem cell deficiency (LSCD), which is characterized by corneal neovascularization, conjunctivalization, and inflammation. Treatment strategies for LSCD include limbal epithelial stem cell transplantation. The fate of stem cells after transplantation remains an unanswered question surrounding this procedure. There are various reports on stem cell tracking after transplantation in LSCD such as DNA fingerprinting, sex mismatch labeling, and green fluorescent protein labeling. The limitations of the existing literature on limbal stem cell tracking include short follow-up periods, the absence of a long-lasting label, ex vivo rather than in vivo labeling, and experiments restricted to animal models such as mice, rats, and goats. This review summarizes the current treatments available for LSCD, the substrates used during limbal stem cell culture and transplantation, and findings from posttransplant, cell tracking experiments conducted up to date.

2. LSCs

The border between the cornea and sclera is known as the limbus. The corneal limbus contains the palisades of Vogt, which harbor the limbal stem cell population (Figure 1). The undulated appearance of the limbal region reflects the morphology of the palisades of Vogt, a series of fibro vascular ridges at the corneal periphery that harbor LSCs. LSCs are localized to the superior and inferior regions of the limbus, which are protected by upper and lower eyelid coverage, respectively (Davanger and Evensen, 1971). The limbal epithelial stem cell hypothesis, described previously, was derives from the X, Y, Z hypothesis of corneal epithelial maintenance (Thoft and Friend, 1983). This model posits that proliferation of the basal cells (X) and centripetal movement of the peripheral cells (Y) help in the homeostasis of the cells shed from the corneal surface (Z). The equation given below explains the hypothesis:

X = proliferation of the basal cells;
Y = the cells from the periphery of the cornea;
Z = the cells shed from the surface;

$$X + Y = Z$$

LSCs have the capacity to self-renew, divide, and differentiate into corneal epithelial cells. As they differentiate, the stem cells successively transform into early transient amplifying cells, late transient amplifying cells, post mitotic cells, and finally into terminally differentiated cells. The capacity of LSCs to self-renew maintains the cell population of the limbus, thus preserving its ability to function as a barrier against encroachment by conjunctiva cells and vascularization of the cornea. The niche where LSCs are located helps in the stemness maintenance of the cells.

2.1. LSC niche

The distinguishing feature of the limbal stem cell niche is its transparency. The limbus is the only stem cell niche that can be visualized noninvasively, through slit-lamp and in vivo confocal microscopy (IVCM), due to its superficial anatomical location. LSCs are deeply buried within the basal interpalisade epithelial papillae of the palisades of Vogt, which in turn are surrounded by melanocytes and covered by pigmented caps of melanocytes. Findings from numerous studies suggest that melanocytes protect LSCs against oxidative DNA damage and ultraviolet radiation (Goldberg and Bron, 1982; Ljubimov et al., 1995; Bessou-Touya et al., 1998; Echevarria and Di Girolamo, 2011). Structurally, the palisades of Vogt provide extra surface area for the accommodation of a large number of LSCs, enabling them to communicate through cell-to-cell, cell-to-extracellular matrix, and paracrine signaling. The palisades of Vogt are highly innervated and richly vascularized. Apart from epithelial stem cells, the limbal niche contains neural crest-derived cell types such as melanocytes, mesenchymal-like stromal

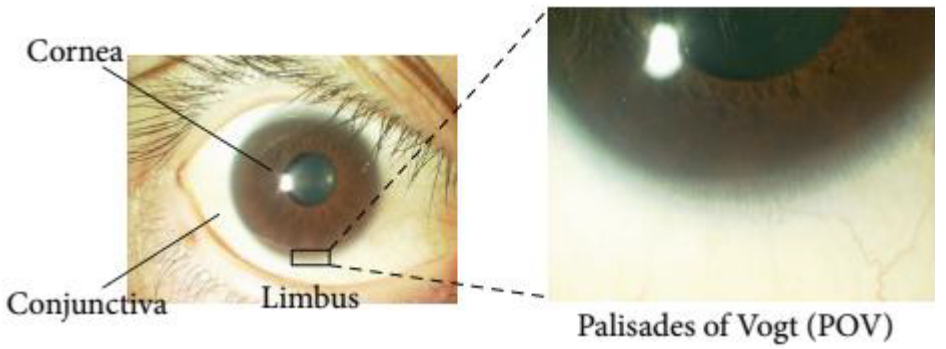

Figure 1. Palisades of Vogt. A slit lamp examination reveals the limbus-specific feature, the palisades of Vogt (Oie and Nishida, 2013).

keratocytes, sensory neurons, vascular endothelial cells, and Langerhans cells for immune surveillance (Gage et al., 2005; Yoshida et al., 2006; Hayashi et al., 2007; Li et al., 2007; Niederer et al., 2007; Li et al., 2012).

Studies of the niche environment in both suspension and explant ex vivo cultures indicate that the niche plays a major role in maintaining the health and stemness of LSCs. In explant cultures the niche is intact and cells migrate outwards to the leading edge, where they are activated through cell cycle control mechanisms in the event of acute injury. In suspension cultures, where the native niche is destroyed, a heterogeneous population of cells forms spheres, which act as a make-shift niche to protect the LSCs in culture (Ksander et al., 2014). In high melanin containing individuals, stripes resulting from melanin migration aid in the identification of LSCs located in the basal cell layer of the limbus. Melanocytes themselves can be used as a substrate to expand the LSCs in both 2D and 3D in vitro cultures. The resulting cells are positive for the expression of putative stem cell markers. The coculture of limbal epithelial cells and human limbal melanocytes (hLM) on Real Architecture For 3D Tissue (RAFT) revealed the formation of multiple epithelial layers and the preservation of basal cells in an undifferentiated state, suggesting that melanocytes play a significant role in stem cell niche regulation (Dziasko et al., 2015).

2.2. LSC marker

Despite the encouraging clinical data on LSC transplantation, a full biological characterization of LSCs is still lacking. Moreover, specific markers allowing for the absolute, prospective identification of LSCs from adjacent nonstem cells or other progenitor cells have yet to be identified. Current data suggest that the expression of biological markers differs between LSCs and other progenitor cells (early transient amplifying cells, late transient amplifying cells, postmitotic cells). Key LSC markers include the extracellular (EC) proteins ABCG2 and Notch-1, and the intracellular (IC) proteins ΔNp63α, C/EBP-δ, Bmi1, and K15 (Pellegrini et al., 2011). In contrast, transient amplifying cells possess the EC markers α9 integrin, β1 integrin, connexin 43, and the IC marker α-enolase. Limbal basal cells express numerous cytokeratin proteins including K5, K15, K14, K19, as well as vimentin (Schlötzer-Schrehardt and Kruse, 2005; Secker and Daniels, 2009; Notara et al., 2010). Among the markers mentioned, expression of ABCG2 is not confined to LSCs, but occurs across limbal basal cells and cytokeratin proteins K5, K15, K14, and K19 are also expressed by mature cornea and conjunctiva epithelial cells (Ohyama et al., 2006; Nubile et al., 2013).

Current research efforts aim to identify a more comprehensive list of markers characteristic of LSCs and progenitor cells. For example, the transcription factor of the Wnt signaling system, TCF-4, has been shown to play an essential role in LSC maintenance via the survivin, p63, p57 signaling pathway (Lu et al., 2012). Periostin, a nonstructural matricellular protein, also called osteoblast-specific factor-2, is expressed only in the basal layer of the limbal epithelium. Periostin colocalizes with well-known putative stem/progenitor markers p63, Bmi-1, and TCF4 (Qu et al., 2015). However, periostin has not been found to colocalize with ΔNp63α.

Other possible LSC markers include primary cilium expression in the presence of large heterochromatinized nuclei, as well as stage-specific early embryogenic antigen-4 and p63α co-expression (Mariappan et al., 2014). Cytoplasmic expression of the leucine-rich repeat-containing G-protein-coupled receptor 5 is found in 100% of cells within the limbal crypt under normal conditions, and expression decreases in the event of inflammation (Curcio et al., 2015). Finally, the ATP-binding cassette subfamily B member 5 (ABCB5) may play an essential role in limbus function, corneal development, and repair. ABCB5 colocalizes with p63α and its variant ΔNp63α in the limbal palisades of Vogt. Label retention assays involving limbal epithelial cells have shown that ABCB5 is expressed in the label-retaining cells presumed to be stem cells (Ksander et al., 2014).

3. LSCD

LSCD is a condition in which the stem cell and barrier functions of the limbus fail (Ahmad et al., 2010). LSCD results in painful blinding due to conjunctivalization, neovascularization, and inflammation of the cornea. The causes of LSCD are broadly categorized into primary (hereditary) and secondary (acquired). LSCD can affect one eye (unilateral) or both eyes (bilateral) and it can be total or partial (Kolli et al., 2010). Table 1 summarizes the etiology of LSCD.

3.1. Current treatment procedures for LSCD

Currently there is no universally approved protocol for the treatment of LSCD. The transplantation procedures listed in Figure 2 vary with regards to cell source, cell type, and culture substrate. A large cohort of LSCD patients have undergone cultivated limbal epithelial transplantation (CLET), which involves the use of human amniotic membrane (hAM) as a culture substrate or scaffold (Sangwan et al., 2011). CLET can occur via two different culture methods: explant or suspension. Explant culture requires the placement of individual segments of a limbal biopsy onto hAM, whereas suspension culture requires enzymatic or mechanical digestion of biopsy tissue to isolate a suspension of LSCs for expansion on USFDA-approved, NIH 3T3-J2 feeder cells (Sangwan et al., 2006; Eslani et al., 2012; Ramachandran et al., 2014). When LSCD is unilateral, the contralateral (unaffected) eye is used as a

Table 1. Etiology of limbal stem cell deficiency.

Primary (hereditary)		Secondary (acquired)	
➢	Aniridia	➢	Contact lens wear
➢	Keratitis associated with multiple endocrine deficiency	➢	Chemical or thermal burns
➢	Ectrodactyly-ectodermal clefting syndrome	➢	Inflammatory diseases like Stevens–Johnson syndrome, neurotrophic keratopathy, bullous keratopathy, Mooren ulcers, etc.
➢	keratitis-ichthyosis-deafness syndrome	➢	Systemic diseases like diabetes, graft versus host disease, rosacea, vitamin A deficiency, etc.
➢	dyskeratosis congenita		

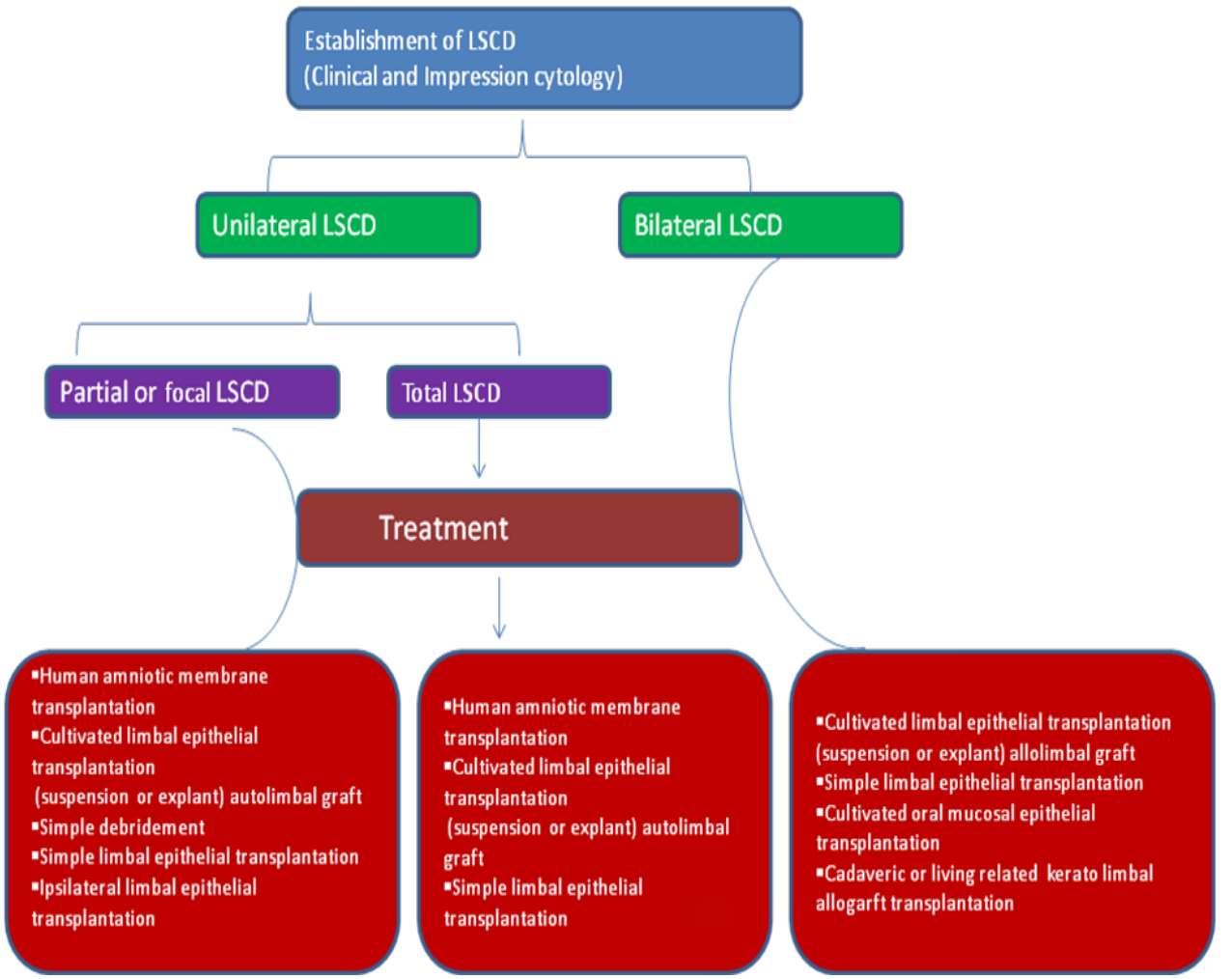

Figure 2. Flowchart for strategies of limbal stem cell transplantation.

cell source. If viable tissue remains despite damage to the eye, cells can be collected from the ipsilateral (affected) eye for treatment (Vazirani et al., 2014). However, bilateral LSCD necessitates an alternative cell source such as oral mucosal epithelial stem cells, conjunctiva epithelial cells, embryonic stem cells, dental pulp stem cells, mesenchymal stem cells from bone marrow, umbilical cord stem cells, or hair follicle stem cells (Homma et al., 2004; Nakamura et al., 2004; Ono et al., 2007; Ang et al., 2010; Gomes et al., 2010; Meyer-Blazejewska et al., 2011; Reza et al., 2011).

The stem cell-based treatment procedures for LSCD have been reviewed by Sevim and Acar (2013).

There are multiple substrates or scaffolds that can be used for the cultivation and transplantation of LSCs (Table 2). Currently, the preferred scaffolds or substrates for limbal stem cell cultivation are the hAM and fibrin gels. The hAM is favored because it provides the stromal network needed for epithelial cells (Malak et al., 1993), secretes essential growth factors (Koizumi et al., 2000), and is less immunogenic (Akle et al., 1981; Adinolfi et al., 1985; Houlihan et al., 1995). Despite having these advantages, the amniotic membrane preparation is a tedious and costly process. These factors encouraged research on other substrates that can be easily prepared in cost effective ways.

Despite the different culture methods and substrates, the success rate of LSC transplantation decreases as the follow-up period increases. The exact mechanism of how the transplanted cells help in curing LSCD is still unknown. There are three possible mechanisms of healing by LSC transplantation:

I. The transplanted stem cells migrate to reach the palisades of Vogt or limbal crypts in the wounded eye.

II. The transplanted stem cells or heterogeneous cell population are able to salvage and activate remaining stem cells in the damaged eye of the recipient.

III. The transplanted stem cells receive and transmit wound-healing signals from the surrounding environment and are fully eradicated after some time. Only recipient stem cells help in the maintenance of corneal homeostasis.

3.2. Need for the labeling and tracking of LSCs

Controversy surrounds the mechanism by which transplanted LSCs repair damage to the cornea. Detailed in vivo studies are needed to monitor the fate of transplanted cells, including their distribution, differentiation, and longevity over time. Previous studies have attempted to label and track the activity of LSCs in the transplant environment. However, due to limitations in current labeling techniques, these experiments did not implicate a definitive mechanism by which the cells facilitate wound repair. Thus, there is a pressing need for the development of a more optimal label for long term monitoring, and particularly one that can be imaged noninvasively in both in vivo and in vitro conditions.

Accurate labeling and tracking of transplanted LSCs for an extended period will elucidate the stem cell homing patterns and reveal any changes in cell properties that occur in the recipient. Effectively addressing these questions will require the coupling of enhanced labeling and tracking techniques with a more detailed understanding of LSCD models involving varying degrees of damage, i.e. partial or focal, total, unilateral, or bilateral.

4. LSC labeling and tracking

Clinical and impression cytology findings were used as a grading system to measure the severity of LSCD before and after the transplant procedure. Impression cytology, however, is invasive to the eye. Thus, there is a need for another method to assess the efficacy of LSC transplantation. IVCM may be a suitable alternative; studies have shown that IVCM and impression cytology analysis of ex vivo cultured limbal stem cell transplant procedures yield comparable results.

A study involving 6-month and 1-year follow-up of keratolimbal allografts have shown that IVCM is a useful tool for monitoring cell density and diagnosing graft failure (Hong et al., 2011). Furthermore, IVCM can differentiate corneal cells from conjunctival cells based on morphological and physiological characteristics and it can demarcate the corneal-to-conjunctival epithelial transition in vivo (Pedrotti et al., 2015). Neither of these studies provides evidence supporting the reformation of the native limbal niche within the palisades of Vogt after transplantation. Limitations of IVCM include the inability to distinguish stem cells from the larger cell population,

Table 2. Summary of substrates used in LSCD treatment.

S. No.	Substrate	Application
1	Human amniotic membrane (Schwab et al., 2000; Tsai et al., 2000; Sangwan et al., 2003)	Clinical
2	Fibrin (Rama et al., 2001)	Clinical
3	Soft contact lens (Di Girolamo et al., 2009)	Clinical
4	Corneal stroma (Espana et al., 2003)	Research
5	PNIPAAm grafted surface (Nishida et al., 2004)	Research
6	Recombinant human collagen hydrogel (Dravida et al., 2008)	Research
7	Silk fibroin (Chirila et al., 2008; Lawrence et al., 2008)	Research
8	Nanofiber scaffold (Zajicova et al., 2010)	Research

and donor cells from recipient cells. The employment of labeling techniques, for example green fluorescent protein tagging, would aid in the identification and tracking of stem cells during IVCM (Shortt et al., 2008).

Stem cell labeling can be indirect or direct. Indirect labeling involves the genetic modification of cells to incorporate the reporter gene with selective expression. By this method, labeled cells can be traced over the long term due to the stable transfection of cells with the reporter gene. Direct labeling requires staining or tagging cells with dyes, fluorochromes, or fluorophores. Although direct labeling is a far less tedious method, it cannot effectively track cells in vivo. Indirect labeling is more suited for in vivo tracking, but the genetic modification requirements make it an impractical technique for use in humans.

Most tracking studies have been conducted in allogeneic transplants (allotransplantation). One allotransplantation study used impression cytology and DNA fingerprinting analysis to determine the fate of transplanted cells and the nature of the cells directly responsible for wound healing during long term follow-up (Henderson et al., 2001a). Follow-up using DNA fingerprinting analysis shows that superficial corneal epithelial cells are those of the recipient rather than the donor, meaning that the transplanted cells are not directly repopulating the damaged epithelium (Henderson et al., 2001b). However, in this case the tracking method used was only able to indicate the origin of the cells, not the mechanism by which the regeneration of the cornea occurred. In a different study, involving sex mismatch limbal allograft transplantation, X, Y chromosome analysis and HLA origin analysis suggested that there is a broad range of cell spectrum in the cell origin (Shimazaki et al., 1999). Impression cytology analysis, performed on patients who underwent cultured limbal epithelial allotransplantation, shows that the donor cells were no longer detectable after 9 months (Daya et al., 2005).

Labeling goat LSCs using the p-Venus vector carrying green fluorescent protein is a new method to track cells after autologous transplantation (autotransplantation) in vitro and in vivo. Venus labeling of cells has shown that donor cells remained in the superficial corneal epithelium for 3 months after transplant, rather than penetrating into the suprabasal or basal layers (Yin et al., 2013). This finding indicates that the transplanted LSCs are not able to reach their native location, the basement membrane, which is well-protected. The short follow-up period and mere single-confirmation of green fluorescent protein expression by polymerase chain reaction may have influenced these results.

Quantum dot labeling has also been utilized to track limbal epithelial cells in vivo. Recent experiments suggest that quantum dots are nontoxic and do not affect cell proliferation or differentiation (Genicio et al., 2015). However, tracking in this study was conducted for a period of only two weeks, and in rabbit rather than human corneas. Nevertheless, standardization of a protocol for quantum dot labeling in human limbal cells is currently underway, as well as for clinical imaging techniques to track the cells. The role of transplanted cells in the long term maintenance of the corneal epithelium through engraftment, positioning, division, movement, and differentiation was not determined in this recent study (Di Girolamo, 2015). Therefore, an urgent need remains for new imaging techniques or combinatorial approaches to tracking human LSCs in vivo to improve the success rate of stem cell transplantation.

5. Conclusion

Limbal stem cell transplantation represents the current best practice for LSCD treatment. Yet, LSC transplant procedures have been only able to cure the disease temporarily, not permanently. Proper biological characterization of LSCs, clarification of the role of the limbal microenvironment, and determination of the fate of LSCs following transplantation are critical to improving the quality and efficacy of LSCD treatment. Currently, there is no "perfect" marker for limbal stem cell identification, which is one principal reason for the shortage of tracking studies, the other being the inadequacy of current labeling methods. The recently identified marker, ABCB5, may enable future studies on LSC tracking after transplantation. Multicolor lineage labeling methods and cell tracking techniques used in other human tissues will also be advantageous to this end. Further studies are inevitably needed to elucidate the mechanism of corneal healing after stem cell transplantation.

The long term efficacy of limbal stem cell transplantation depends upon the isolation and maintenance of the stem and niche cells for grafting in an organized 3D scaffold or substrate. In order to track cell fate in vivo, labeling techniques employing harmless genetic modification and fluorescent reporter protein expression would be preferred. The long term, stable expression of a fluorescence reporter in transplanted LSCs may be the key to elucidating the mechanisms behind corneal regeneration in LSCD patients. Successful methods for tracking stem cells in other parts of the human body may be applied to LSC tracking after transplantation. Fluorescein iso thiocyanate conjugated mesoporous silica nanoparticle labeling (Huang et al., 2005), cholera toxin B conjugated quantum dot (CTB-QD) labeling (Villa et al., 2010) and bioluminescence imaging (Huang et al., 2012) represent a few of these potential techniques. Moreover, technological advances enabling lineage tracing and single cell analyses in hematopoietic stem cells may also be applicable to LSCs (Nimmo et al., 2015).

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