

Clinical applications of limbal epithelial stem cells

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Abstract

The cornea is the protecting and refractive part of the eye essential for proper visual acuity. Limbal epithelial stem cells (LESC) are responsible for maintaining the homeostasis of the corneal epithelium. They reside in the limbal stem cell niche within the transition region between the cornea and the sclera, the so-called limbus. The characterization of the stem cell microenvironment in terms of molecular regulation and cytoarchitecture remains the area of intensive research. Recently, novel niche structures were proposed: limbal epithelial crypts (LEC), limbal crypts (LCs) and focal stromal projections (FSPs). To date, a variety of limbal stem cells markers have been suggested but no definitive one has been identified. Limbal stem cell deficiency (LSCD) is a pathologic condition caused by destruction or dysfunction of LESE. This state may occur in a number of hereditary and acquired diseases and lead to pain, reduced visual acuity, and even blindness. This review will briefly outline the current state of knowledge on limbal stem cells biology and the role of transplanted stem cells as potential tools in regeneration of the cornea.

Key words: cornea, limbus, stem cells, transplantation.

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Limbal epithelial stem cell niche

The cornea is the unvascularised refractive element of the eye responsible for approximately two-thirds of its total optical power. Its integrity and transparency is essential for proper functioning of the visual system. The cornea consist of five layers: the outermost stratified squamous epithelium, the Bowman's membrane, the stroma accounting for about 90% of its thickness, the Descemet's membrane and the innermost endothelium actively pumping water out of the stroma. The limbal epithelial stem cells (LESCs) reside in the basal layer of the transition region between the corneal and conjunctival epithelium which is referred to as the limbus [1, 2]. The stem cell niche is considered to be located in the limbal palisades of Vogt which are radially pointed stromal ridges intersected with epithelial rete pegs more abundantly present in the superior and inferior part of the limbus [3, 4]. The term "palisades of Vogt" was coined approximately two centuries ago while observing these radial linear structures under the slitlamp. Such a location provides LESEs with protection by the lids and blood supply from near vessels [5]. Differences in the

composition of the limbal basement membrane in comparison to the rest of the cornea are considered to play a mechanical protective role for resident stem cells [6, 7]. Furthermore, melanin pigmentation safeguard LESEs from potential damage by UV light [8, 9]. The limbal zone as opposed to the central cornea has distinguishing features, such as the thicker epithelium which forms pegs consisted of 10-12 layers and absence of the Bowman's layer and the Descemet's membrane [10]. Recently, novel components of the stem cell niche were proposed: limbal epithelial crypt (LEC) [11], limbal crypts (LCs) and focal stromal projections (FSPs) [12]. In support of this findings is observation that LCs and FSPs don't occur in patients with limbal stem cells deficiency (LSCD) and cells within them express high levels of the putative limbal stem cell markers p63 α and ABCG2. These structures are predominantly distributed in the superior and inferior corneal limbal quadrants and extend to a varying degree temporally and nasally with no presence in the horizontal meridian. An advance in technology of the observation and identification *in vivo* stem cells niches is essential in terms of improvement in stem cells harvesting by the targeted

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biopsy. Understanding the molecular mechanisms controlling the stem cell microenvironment remains a major challenge in the area of stem cells research [13, 14]. So far, the role of basement membrane heterogeneity and cytokines in limbal stem cells differentiation is still elusive.

Limbal stem cell concept

Davanger and Evensen were the first to propose that the corneal epithelium was renewed from a source of cells located at the limbus [1]. They observed that pigment in the epithelium in heavily pigmented eyes migrated in lines from the limbal region to the central cornea while healing. In contrast to these studies are recent findings that in the mouse the central corneal epithelium contains oligopotent stem cells [15]. The limbal stem cell concept is strongly supported by the observation that slow cycling cells which can be identified experimentally as “label-retaining cells” (LRC) are restricted to the limbal basal layer [16]. Further evidence in support of the limbal-stem cell hypothesis is provided by experiments showing that LESC have a greater both *in vitro* [17] and *in vivo* [18] proliferative potential than corneal epithelial cells. Additional evidence comes from the pioneering studies by Tseng *et al.* showing that LESC transplantation can result in persistent restoration of the entire corneal epithelium [19]. In addition, all corneal epithelial neoplasias are associated with the limbus [20, 21]. The putative LESC have a high nucleus/cytoplasm ratio, heterochromatin rich nuclei with no well-defined nucleoli [2, 5, 22] and smaller size as opposed to basal epithelial cells of the central and peripheral cornea [23]. The limbus is a unique model for the study of adult stem cells due to its accessibility and the fact that limbal stem cells are well physically separated from transient amplifying cells (TA) [24].

Corneal epithelial cells turnover

Astonishingly, the cornea is an exception among stratified epithelia because it has evolved differently [25]. It doesn't contain stem cells but relies on migration of transit amplifying (TA) cells which are generated by occasional asymmetrical division of LESC giving rise to stem cells and daughter TA cells [26]. A population of LESC is responsible for replacing terminally differentiated cells by providing a constant supply of cells that replace those removed from the cornea. Lehrer *et al.* demonstrated that there are three strategies to expand the corneal epithelium cell population upon wounding: the recruitment of stem cells to produce more TA cells, the increase in the number of times the TA cell can replicate, and the shortening of the cell cycle time [27]. Unlike the epithelium, cell turnover of the stroma is very slow and the endothelial layer is thought to lack the capacity to regenerate. However, in 2005 this notion has been challenged by Whitehart *et al.* who demonstrated mitotic activity by peripheral corneal endothelial cells [28].

Limbal stem cell deficiency (LSCD)

Limbal stem cell deficiency (LSCD) can occur in a variety of hereditary or acquired disorders including chemical or thermal injury, ultraviolet and ionizing radiation, Stevens Johnson syndrome, advanced ocular cicatricial pemphigoid, contact lens-induced keratopathy, multiple surgeries or cryotherapies to the limbal region, aniridia or extensive microbial infection [26, 29]. LSCD can be classified as partial with deficiency of LESC limited to a certain region of the limbus and as total with conjunctivalization of the entire cornea. Clinically, this leads to epithelial haze, superficial subepithelial vascularization, persistent or recurrent epithelial defects, epithelial and stromal inflammation, late fluorescein staining and loss of the limbal palisades of Vogt [5, 29]. Patients suffer from photophobia, discomfort, reduced visual acuity and even blindness [26]. The key element to the diagnosis of LSCD is conjunctivalization – process of conjunctival epithelial cells and blood vessels migration onto the corneal surface [30]. Impression cytology may be performed to identify conjunctival goblet cells using periodic acid Schiff stain or to confirm a conjunctival phenotype using monoclonal antibodies to cytokeratin 3 and cytokeratin 19 [31].

Transplantation of *ex vivo* cultured LESC

Traditional techniques used to transplant LSCs include keratolimbal lamellar allograft (KLAL), conjunctival-limbal autografts (CLAU), living-related conjunctival-limbal allografts (lr-CLAL) or oversized and eccentric penetrating keratoplasties [31]. In humans Pellegrini *et al.* were the first to describe the use of *ex vivo* cultured LESC for therapeutic applications [32], hence ophthalmology was placed at the forefront of stem cell research. This technique is based on a minimal limbal biopsy performed in contralateral healthy eyes (in case of partial LSCD) and living related donors or fresh cadaveric corneas (in case of total LSCD). The next step is an isolation and *ex vivo* culture of LESC to produce a sheet of stem cells for transplantation. Theoretical advantages over traditional transplantations include minimizing the risk of stem cell failure in the donor eye and allograft rejection by the absence of antigen-presenting Langerhan's cells in the graft. There are two main methods of producing *ex vivo* cultured LESC for transplantation, the explant culture system and the suspension culture system with some modifications of both techniques described [31, 33]. In most current approaches, the amniotic membrane, paraffin gauze, collagen shields, fibrin gels, temperature sensitive biopolymers and even the human anterior lens capsule are used as a substrate for LESC growth [34, 35]. The major disadvantage of current culture methods is the use of animal derived products in the culture media. A replacement of the fetal calf serum (FCS) with the autologous serum from the patient represents the reduced

but still remaining risk of possible infection [31, 36]. However, recently Di Girolamo *et al.* managed to overcome this problem in 3 patients with LSCD by the use of a contact lens-based technique for expansion and transplantation of autologous epithelial progenitors [37]. A study by Daya *et al.* based on polymerase chain reaction (PCR) genotyping of cells populating the ocular surface postoperatively indicates that stem cells can persist up to 9 months post-transplantation and therefore their role is probably limited to creating the niche for host stem cells [38].

Putative LESC markers

To date, several putative stem cell markers have been proposed, however no single molecular one has been identified. Therefore a combination of these markers with the non-expressed ones is used to identify LSCs [39]. Positive LESC markers are cytokeratins Ck15, Ck14, Ck19, NGF receptor TrkA, vimentin, integrins $\alpha 6$, $\alpha 9$, $\beta 1$ and $\beta 4$ [10]. Negative markers include involucrin, connexins 43, 50 and cytokeratins Ck3 and Ck12 [10,40]. Currently, the transcription factor $\Delta Np63\alpha$ which is an N-terminally truncated α isoform of the p63 gene is considered as a reliable marker of both resting and activated LSCs [5, 10, 40]. The ABCG2 transporter protein is also suggested to mark stem cells at the limbus [5, 10, 39, 40]. Recently, C/EBP δ and Bmi-1 are thought to be useful in stem cells identification [10, 39]. In recent years, the use of the high throughput nucleic acid profiling and proteomic techniques allowed to identify several molecules as potential stem cell markers. These include ephrins, cytokeratins 14 and 15, p-cadherin, wnt-4, superoxide dismutase 2 (SOD2) and heatshock protein 70 (HSP70.1) [10, 41-43]. Interestingly, a study by Monteiro *et al.* indicates that human immature dental pulp stem cells express markers in common with LSCs and might be successfully used as a potential alternative source of cells for corneal epithelium reconstruction in rabbits [44].

Future prospects

Regenerative medicine is a promising field of research initiated by ground-breaking work by Howard Green and his colleagues in 1984 [45]. However, a greater understanding of stem cells biology is required to achieve the widespread use of limbal stem cells for therapeutic purposes. The key limitation in LESC research remains the identification of definitive stem cells markers. An advance in imaging techniques opens prospects for a higher success rate of the identification, isolation and *ex vivo* expansion of limbal stem cells. Finding an alternative source of stem cells which can be successfully used in the clinic is one of challenges in cornea regeneration. However, a rapid progress in stem cells research offers hope of restoring vision for patients suffering from LSCD.

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