The Mammalian Limbal Stem Cell Niche: A Complex Interaction Between Cells, Growth Factors and Extracellular Matrix

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1 Introduction

After experiments by Till and McCulloch which led to the experimental evidence that supported—for the first time- the existence of stem cells [1, 2], the search for these cells and their site of residence became one of the major challenges in Cell Biology. At the beginning, people looked for cell populations that showed some of the theoretical characteristics expected for stem cells such as: (i) The expression of early differentiation markers previously identified either during embryonic development as well as in vitro [3, 4]. (ii) The use of assays to quantify the proliferative potential of cells directly isolated from tissues [5–9]; and (iii) the long-term engraftment of presumptive stem cells into injured tissues [10, 11].

After the evaluation of tissues that undergo a continuous replenishment, authors concluded that adult stem cells possess the self-renewal ability through mitotic cell division, a crucial property because of its participation in the creation of new tissues and in the maintenance of the stem cell pool [12, 13]. Moreover, it was predicted that this cell population should have an unlimited proliferative potential while its progeny undergoes differentiation into a wide range of specialized cell types.

On basis of the above properties and considering further studies which demonstrated that stem cells either remain in a quiescent state or slowly progress through cell cycle [14–16], in addition to results which suggested their presumptive localization [15, 16], researchers proposed that stem cells were found in specific anatomic sites. These sites were designated as “niches”.

The concept of a stem cell niche was first proposed by Schofield in 1978 [17], and its definition has evolved during the last forty years. In contrast to its initial meaning which corresponded to the site of residence of stem cell populations in

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animal tissues, the concept has changed following an equivalent evolution to its use in Ecology. Indeed, the niche was described as the role of stem cells in a developmental web and more recently as a restricted tissue microenvironment, in reminiscence of definitions of ecological niches by Grinnell [18] and Elton [19], respectively.

Currently, based on the different interactions that presumptive stem cells maintain with environment, stem cell niche definition is closer to the Hutchinsonian niche where environmental conditions and resources define the requirements of an individual or a species [20]. According to such characteristics, stem cell niche may be described as an anatomically defined and protected location that provides housing, positioning information and signaling inputs necessary to support normal stem cell activity [reviewed in 21].

By the use of different experimental approaches, such as retention of DNA precursor analogs [14, 16, 22, 23], the presence of specific surface antigens or the expression of specific adhesion molecules [3, 4, 24], and the lack of terminal differentiation markers [4], it has been possible to propose the location of presumptive stem cell niches for epithelial tissues as epidermis [4, 14, 16, 22–25], esophageal [26], urothelium [27, 28], bronchiole-associated [29], and conjunctival [30–32], among others.

In contrast with epidermis, the stem cell niche for corneal epithelium was not a clear entity. For a long time, corneal wound healing after injury was considered an outcome of conjunctival epithelial cell migration and transdifferentiation into corneal epithelial cells [33–37]. However, the incomplete and reversible conversion of conjunctival cells into corneal epithelium, the recurrent erosions observed in conjunctivalized corneas [30–38], and the discovery of the limbus as the supposed location of corneal epithelial stem cells, led to reject the conjunctiva as a source of cells for corneal epithelial renewal and healing.

The following paragraphs will review the characteristics of the corneal stem cell niche, the cell populations comprised in its structure, as well as the extracellular matrix and signaling pathways that compose, regulate and maintain stemness in adult corneal epithelium.

2 Defining the Limbus as the Residence Site for Corneal Stem Cells

Adult stem cells reside in specialized, protected sites in many organs and differentiated tissues. The majority of adult stem cells are “tissue-specific”: they have the ability of self-renewal and they only differentiate into those cell types that compose the organ used as site of residence. So far, accumulated evidence suggests that nichesregulate the number and frequency of stem cell divisions, and the number of committed daughter cells; however, the homeostatic mechanisms involved in such regulation are still unclear and depend on the structure and regeneration needs of the tissue.

For a better understanding of the structural, functional and regulatory characteristics of stem cell niches, and their participation in tissue homeostasis and repair, the ocular surface epithelium provides an exceptional model, thanks to its accessibility, a defined anatomy and the presence of a well-defined stem cell niche.

As previously said, for many years conjunctival epithelium was considered the possible source of corneal epithelial cells, as suggested by results from different laboratories [34–38]. Nevertheless, Davanger and Evensen [39] studied the papillary structure located at the human limbus, and made wound-repair experiments in guinea pigs, leading them to speculate that corneal epithelial renewal depended on the limbus. Afterwards, immunostaining of eye surface and corneal cell cultures with monoclonal antibodies raised against the corneal-specific keratin K3 provided the first experimental evidence that suggested the specific location of corneal epithelial stem cells at the basal layer of the limbal epithelium [40]. This breakthrough rapidly led to a series of experiments that provided further support for the limbal location of corneal stem cells: mainly the lack of the K3/K12 keratin pair in limbal basal cells [40–42], and the existence of label-retaining cells at this location [43]. Later, such evidence was strengthened by studies showing that limbal basal cells have a higher proliferative potential than central corneal basal cells [31, 44–46], and they show a differential response to chronic stimulation with phorbol esters [47]. Moreover, limbal epithelial cells have a greater ability to grow in colony forming assays [46], serving as founders of holoclones similar to those described for epidermis [48] and hair follicle [49] (see Fig. 1).

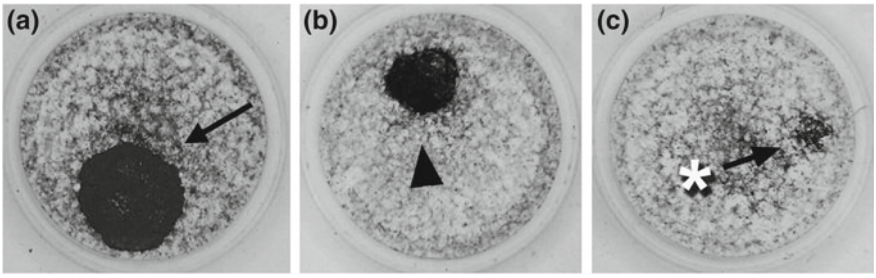

Fig. 1 Example of the three types of clonal growth shown by corneal epithelial cells. Beginning with a cell suspension obtained after dissociation of a primary culture, corneal epithelial cells were plated into 2.0 cm^2 wells (24 multi-well plates). For plating, cells were diluted up to a density of 1 cell/ml; by the use of a glass pipette one cell was placed into one culture well, previously inoculated with 2.2×10^4 cells/cm^2 mitomycin C treated 3T3 feeder cells. After 10 days, colonies were formalin-fixed and stained with Rhodamine B, as previously described [248]. The figure shows typical colonies formed by the isolated corneal epithelial cells. **a** Holoclone (*arrow*), which is a large colony with a smooth perimeter, formed by small cells and few differentiated cells [48]. After dissociation, holoclones generate more holoclones. **c** Paraclone (*asterisk*), which shows low or null proliferative potential. They are composed by differentiated cells [48]. Finally **b**, a Meroclone (*arrowhead*), which is a clone of mixed composition and gives rise to paraclones with high frequency [48]

Given that, differentiation-linked keratins are not useful to isolate stem cells because they are late intracellular markers associated to terminal phenotype [41, 42, 50, 51], the postulation of limbus as the presumptive site of residence of corneal stem cells prompted the quest for specific molecules useful to detect, separate and characterize the basal limbal epithelial cells. These studies allowed the recognition of molecular markers that revealed some of the regulatory, metabolic, proliferative and adhesive characteristics of corneal stem cells, besides their possible use as tools to isolate this cell population.

In many instances, researchers looked for molecules suggested as stem cell markers in other epithelia. For example, p63, a transcription factor previously proposed as a molecular marker of epidermal stem cells [52, 53], showed a restricted distribution to the limbal epithelium suggesting its participation in cell proliferation [54, 55]. More specifically, p63 isoforms (mainly ΔNp63α) seems to regulate the proliferative and migratory potential of limbal [55–59] and corneal epithelial cells [58, 59], establishing a corneal gradient in which ΔNp63α levels peak at the limbus [56–59]. A similar situation was described for TCF4, crucial for the Wnt signaling transduction pathway, which together with β-catenin participate in the maintenance and survival of stem cells [60, 61].

Among proteins that can be used to identify corneal stem cells, we find metabolic enzymes such as α-enolase [62, 63], and the cytoskeletal elements vimentin and K19 keratin [64]. Interestingly, vimentin intermediate filaments, considered typical of mesenchymal-derived cells [64–66], have been observed during epithelial-mesenchymal transition (EMT) [67] and might be involved in migration of epithelial cells that express some stem cell markers [68] as demonstrated for epidermal keratinocytes. Moreover, membrane proteins such as $\alpha_9\beta_1$ integrin which is a receptor for Extracellular Matrix (ECM) components involved in corneal epithelial cell adhesion and migration [69–71], shows a specific association with limbus and may contribute to the identification of corneal stem cells [70–73].

Other criteria used to identify the site of residence of stem cells depend on the physiological behavior of this cell population. Existing evidence suggesting that adult stem cells remain in a quiescent state, or that they slowly progress through the cell cycle, came from experiments in which corneal tissue was exposed to long labeling periods with DNA precursors. After a label-dilution period, label-retaining cells (LRC) exclusively found at the limbal basal layer, were considered corneal stem cells [43]. In addition, the use of the exclusion of vital DNA binding dye Hoechst 33342 to identify the Side Population (SP) [74] showed that limbal epithelial stem cells express high levels of the ATP binding cassette transporter protein ABCG2 and its corresponding mRNA [75–78], which could be playing a role in protection of stem cells [79].

The expression of these markers [for an exhaustive review see 80] in combination with the use of molecules associated to terminal phenotype, such as the high levels of CD71 [81], SSEA-4 [82, 83] or integrins β1, β4 and α3 [70, 72]; will allow the enrichment of corneal stem cell populations. This will establish a new perspective for the long-term maintenance of stem cells, their analysis, the study of the homing mechanism and their effective use for tissue regeneration in the clinic.

3 The Limbus: Its Structure and Composition

In addition of searching for molecular markers useful for the isolation and characterization of stem cells, different laboratories were involved in the analysis of the limbal microenvironment. Soon, it was proposed that the limbus possesses anatomical and functional dimensions that participate in the maintenance of "stemness". In contrast with the central corneal epithelium which lays on an extremely flat corneal stroma with no rete ridges, the limbus is covered by a stratified epithelium in which basal cells are smaller and more closely packed, showing a series of interdigitations with limbal stroma, and constituting a serrated basal side (Fig. 2) [84]. Moreover, the limbus is characterized by stromal invaginations known in humans as the Palisades of Vögt. These are papillae-like projections, which show a distinctive vasculature with radially oriented arterial and venous components [85]. So, the Palisades of Vögt were suggested as the reservoir which: (i) protects the stem cells from traumatic and environmental insults, (ii) allows epithelial-mesenchymal interactions, and (iii) provides access to chemical signals that diffuse from the rich underlying vascular network [39, 86–88].

Later studies showed that the limbus contains a specific anatomical structure which probably provides the microenvironmental characteristics that correspond to the stem cell niche. This structure was termed the Limbal Epithelial Crypt (LEC) [89] or Limbal Crypt (LC) [90], and consists of a cord or finger of cells that invaginates into the limbal stroma from the rete ridges located between the palisades, and extends radially into the conjunctival stroma [89, 90]. Some characteristics of this anatomic structure were part of the evidence that suggested that corneal stem cells reside in there: mainly the expression and content of cytokeratin K14, which showed a pattern similar to that observed in basal cells at the rest of the limbus, and the highest staining for ABCG2 [89] and p63 [90]. It is important to point out that, limbal crypts have not been found in other species besides humans and pigs [91].

As soon as the possible anatomical location of corneal stem cells was established, major questions emerged. How is the stem cell population regulated? Which nichecomponents maintain stemness? This interrogations are under active research, and attempts to understand limbal basal cell interactions with other cells, extracellular matrix (ECM) components and, growth factors and cytokines, associated to the limbal crypt. These components and interactions concurrently create the microenvironmental conditions equivalent to the Hutchinsonian multidimensional niche [20], that regulates and maintain stemness in corneal epithelium. These interactions might also be involved in the mechanisms that seem to be regulating stem cell homing and migration.

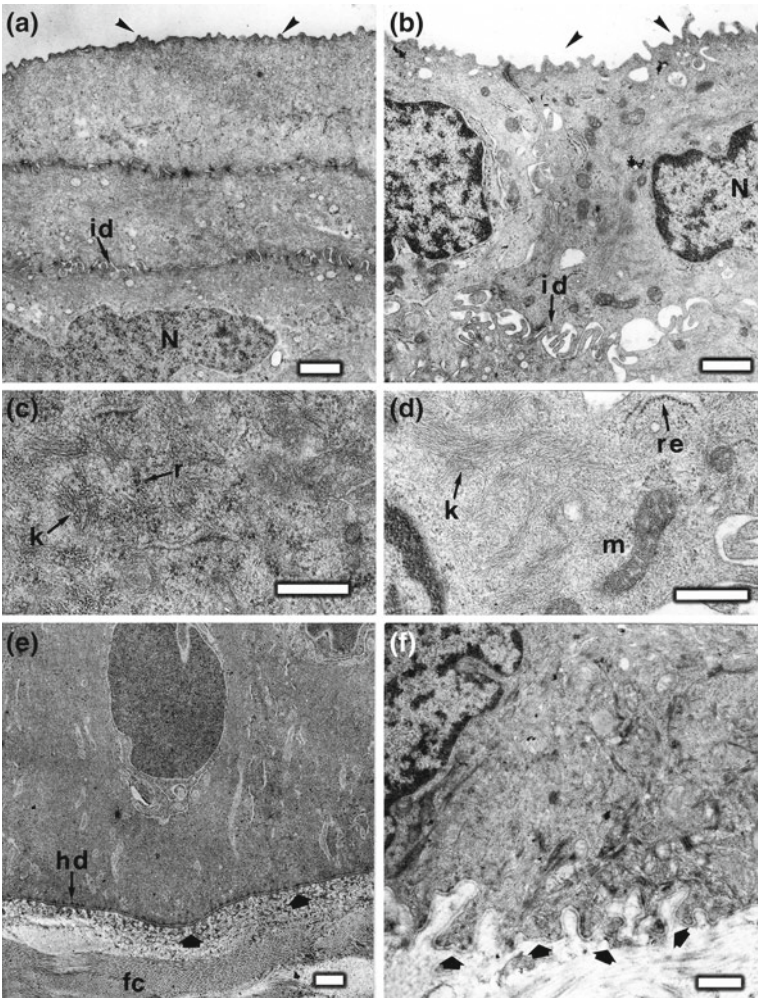

Fig. 2 Comparative ultrastructure of adult rabbit (**a**, **c**, **e**) corneal and (**b**, **d**, **f**) limbal epithelia. While basal cells of corneal epithelium (**e**) rest onto a flat basement membrane, and showed nuclei with a more condensed chromatin, basal cells at the limbal epithelium (**f**), had a lower nucleus/cytoplasm ratio, a less condensed chromatin, and showed a basal side highly interdigitated (*arrows*) with the corneal stroma. **a** Morphology of corneal epithelium superficial cells, with microvilli and microplicae (*arrowheads*) at their surface. **b** Limbal superficial cells were less keratinized a showed a higher number of mitochondria. Similarly, wing cells were more keratinized in cornea than at limbus (**c**, **d**). For (**a**–**c**, **e**–**f**), scale bar = 1.0 μm. In **d**, scale bar = 0.5 μm. *N* cell nucleus; *k* keratins; *id* interdigitations among cells; *r* ribosomes; rough endoplasmic reticulum; *m* mitochondria; *fc* collagen fibers at corneal stroma; *hd* hemidesmosomes

3.1 *Corneal Epithelial Stem Cells and Their Niche: Cells that Interact with the Limbal Stem Cell Reservoir*

Anatomically and physiologically, the limbusconsist in a complex structure proximally delimited by a line that intersects the peripheral extremes of Bowman's and Descemet's membrane and distally by a line that traverses from scleral spur to the tangent of the external surface of the ocular globe. Among its earlier described functions, limbus supplies nourishment for peripheral cornea and participates in maintenance of the intraocular pressure [84, 85].

As it normally occurs in other stem cell niches, the limbus houses different cell populations that participate in the conditioning of the microenvironment for nourishing, protecting, maintaining and regulating self-renewal and fate-decision of the resident epithelial cells. Different studies have recognized that, in addition to the epithelial cells, several other cell types such as stromal cells [92, 93], melanocytes [94, 95], dendritic or Langerhans cells [96] and telocytes [97], are present in the limbal niche. Moreover, at the Palisades of Vögt there is a rich, distinctive vasculature with narrow, radially oriented hairpin loops [98, 99], that participates in such functions, in a similar manner to the association between the vascular niche and the stem cell reservoirs in bone marrow and mouse germinal epithelium [92, 98–100].

3.1.1 Epithelial Cells

At the limbus, epithelial cells constitute a stratified epithelium similar to the one observed in peripheral and central cornea. However, the numbers of limbal cell layers increases to 10 or 15, and superficial cells have rough surfaces unlike the smooth superficial cells in central cornea [84]. In this region, basal cells are more closely packed and smaller, showing a large nucleus/cytoplasm (N/C) ratio [78, 101, 102]. They also express molecular markers associated to epithelial stem cells and showing high growth potential to generate holoclones [46, 103], as expected for stem cells. Nevertheless, basal limbal cells consist of a heterogeneous cell population composed by the stem cells and their progeny, which becomes committed to express the differentiation process after going through a limited number of cell divisions known as Transient Amplification [46, 104]. These transient amplifying cells exhibit intermediate features between stem and committed cells, until the expression of the differentiated phenotype leads to down regulation of stem cell markers [105, 106].

Despite the wide variety of molecular markers described for limbal epithelial cells (see Table 1), their use for the specific selection of the stem cell population has not been as successful as expected. This is a consequence of the persistence of stem cell markers in the transient amplifying cell population and in the early differentiating cells [105, 106]. Therefore, separation of cells by the use of techniques that take advantage of stem cell markers do not only assures the enrichment of stem

Table 1 Some molecular markers distinctive of limbal stem cells

Molecular marker	Limbal expression	Corneal expression	References
Cytoskeleton			
K3 keratin	Suprabasal	Basal and suprabasal cells	[40]
K12 keratin	Suprabasal	Basal and suprabasal cells	[41, 42]
K8 keratin	Basal, in clusters	Not detected	[249]
K15 keratin	Basal, in clusters	Not detected	[249]
K19 keratin	Basal	Not detected	[64]
Vimentin	Basal	Not detected	[64, 250]
Nestin	Suprabasal	Basal and suprabasal	[242]
Metabolism			
α enolase	Basal	Not detected	[62, 63]
LDH isoforms	Low, basal, high suprabasal	High both basal and suprabasal	[250]
$NADP^+$-dependent isocitrate dehydrogenase	Low levels or not detected	Overexpressed in corneal epithelium, except superficial cells	[251]
Adhesion			
Integrin α6	Basal (high levels)	Basal (low levels)	[70]
Integrin α9	basal	Not detected	[72, 73]
Integrin α3	Basal (low levels)	Basal (high levels)	[70, 72]
Integrin β1	Basal (low levels)	Basal (high levels)	[70, 72]
Integrin β4	Basal (low levels)	Basal (high levels)	[70, 72]
β-catenin	Nuclear localization, basal	Basal, membrane-linked	[61]
Nectin-3	Side population	Not detected	[71]
Transcription factors			
ΔNp63α	Basal (high levels)	Basal (low or null levels)	[55–59, 78]
ΔNp63β	Basal (high levels)	Basal (low or null levels)	[57–59]
ΔNp63γ	Basal (high levels)	Basal (low or null levels)	[57–59]
Pax-6	Entire epithelium, although probably lower expression in basal cells	Entire epithelium	[252, 253]
TCF-4	Basal (high levels)	Not detected	[60, 61, 80, 184]
Membrane proteins			
Notch-1 (contradictory information)	Basal (high levels)	Basal (low levels)	[206]
		High levels suprabasal	[171]
EGF receptor	Basal (higher than central cornea)	Basal (high levels)	[242]

(continued)

Table 1 (continued)

Molecular marker	Limbal expression	Corneal expression	References
CD71	Low levels	High levels	[81]
CD61	Side population	Not detected	[71]
ABCG2	High expression, strongly stained	Low expression	[75–79]
SSEA-4	Low or absent	High levels	[82, 83]
Wnt 2	Nucleus	Not detected	[61]
Wnt 6	Present	Not detected	[61]
Wnt 11	Present	Not detected	[61]
Dkk-1	Present	Not detected	[61]
WIF	Present	Not detected	[61]
FRZB	Present	Not detected	[61]
Fz1	Higher in limbus	Lower than limbus	[254]
Fz4	Higher in limbus	Lower than limbus	[254]
Fz10	Higher in limbus	Lower than limbus	[254]
Fz7	Preferentially at basal cells	Not detected	[254]
CLED (calcium-linked epithelial differentiation)	Not detected	Expressed in basal and intermediate cells	[248]
Connexin 43	Not detected in basal, only suprabasal	High expression	[78, 242]

In this table, are shown some of the molecular markers found at limbus, comparing their expression with the one observed in central cornea

cells [107], because the isolated population also includes committed cells that progress through the transient amplification period and generate a set of non-proliferative, terminally differentiated cells [104].

3.1.2 Melanocytes

One of the distinctive traits of the limbal region, besides the Palisades of Vögt, is the presence of melanocytes located at or close to the epithelial basement membrane [84, 103]. Depending on the species and the individual, these cells confer to epithelial limbal cells variable levels of pigmentation [39, 95, 108]. In humans, melanocytes interact with $K19^{+}$/N-cadherin^{+} basal limbal epithelial cells, which show melanin granules at their apical domain acting as a pigmented cap that faces the ocular surface [94, 109], with a distribution similar to that described previously in keratinocytes found at the deep rete-ridges of the skin [22, 23]. These melanocytes are found in a ratio of about 1 for each 10 limbal epithelial cells [94], and it is thought that they might have a role in the protection of the stem cell reservoir from UV radiation and oxidative stress [94, 109].

3.1.3 Langerhans' Cells

In the cornea, the permanent presence of Langerhans' or dendritic cells that represent the professional Antigen Presenting Cells (APCs) of the ocular surface, has been detected mainly at the limbus, peripheral and pericentral cornea [110, 111]. It was demonstrated amongst guinea pig, hamster, mouse, and human corneas [96, 110–112], the existence of a density gradient of mature dendritic cells which show the constitutive expression of the Major Histocompatibility Complex (MHC) class II antigens, and the expression of CD11c and CD45 [112, 113]. Under such density gradient, mature dendritic cells are highly abundant at the limbus and peripheral cornea, while immature cells are predominant at central cornea [96]. Interestingly, it was described that at the limbus, one fifth of the $ABCG2^{+}$ label retaining cells correspond to Langerhans cells [112]. The existence of this dendritic cell population has been related with the immunologic surveillance of the cornea, and with inflammatory processes [96]. In addition, the location of $ABCG2^{+}$/label-retaining dendritic cells at limbus also suggests that a specific subpopulation of Langerhans cells with stem cell characteristics or dendritic cell precursors [96] is maintained by the microenvironmental conditions prevalent at this anatomic site, that also support the stemness of limbal epithelial cells.

3.1.4 Stromal Cells

On the other hand, stromal cells adjacent to limbal stem cells could be crucial for the establishment and maintenance of the limbus as a niche. This proposal was supported by the description of the epithelial cell-filled crypts localized between the limbal palisades of Vogt [89, 90], where stromal cells closely underlie the epithelium [84] resembling the epidermal rete ridges [22, 23] and suggesting a cross-talk between epithelial and stromal cells.

The importance of the stromal cells was supported with experiments demonstrating that these cells could be grouped in two different cell types: limbal stromal cells (LSC) and limbal niche cells (LNC) [92]. After their cultivation and use to support the growth of corneal epithelial cells, it was concluded that LNC possess a higher ability than LSC to maintain stemness and to support the expression of factors that keep up the limbal epithelial stem/progenitor cells characteristics [93]. In addition, LNC enhanced the formation of stratified epithelial cell sheets and the growth of limbal stem/progenitor cells in colony forming assays [93, 113].

Further studies have shown that LNC interact with the basal epithelial cells at limbus [103, 113]. Such interaction seems to be established between adjacent cells [105] and depend on the stromal-derived factor 1 (SDF-1), also known as C-X-C motif chemokine 12 (CXCL12), and its receptor CXCR4 [114]. Since disruption of signaling mediated by SDF-1 leads to loss of holoclone-forming units as well as the disaggregation of cells with proteolytic enzymes, researchers have concluded that maintenance of stem cell phenotype depends upon physical contact between limbal epithelial cells and LNC [114].

The importance of this interaction is emphasized by results from different groups, which have highlighted the need of LNC in order to obtain a better growth and differentiation of the limbal epithelial cells [103, 113, 114]. These results have encouraged the search of methods to grow and expand LNC, oriented to their use for cultivation of limbal epithelial cells for clinical application [114, 115].

3.1.5 Corneal Innervation

An element that could also be essential for the establishment of niche microenvironment consists in the innervation of corneal tissue. Both cornea and limbus are the most densely innervated surface tissues. They are supplied by sensory nerve fibers derived from the ophthalmic division of the trigeminal nerve, via the long posterior and short ciliary nerves [84, 116, 117]. Besides the sensory nerves, corneal and limbal innervation also involves sympathetic branch derived from the superior cervical ganglion [84, 116, 117] and a parasympathetic network which originates from accessory ciliary ganglion neurons [118]. Within this network, epithelial nerve density and the number of nerve endings are higher at the center of the cornea, rather than the periphery [119].

Results from different laboratories have shown that the superficial network that surrounds the limbal area supplies the innervation at limbus and peripheral cornea, while nerves at central epithelium derive from branches of the stromal network [116–119]. Stromal nerves enter into central cornea establishing a radial pattern [119] which is reminiscent of the centripetal migratory pathway followed by corneal epithelial cells from limbus to central cornea which also shows a radial pattern [120, 121].

Therefore, in addition to their important sensory functions, the possible functional roles for innervation in the avascular cornea are not clear. It was reported that signals from the nervous system modulate localization and mobilization of hematopoietic stem cells into the endosteal bone [122], and the migration and proliferation of bulge stem cells in hair follicle [123]. In view of this evidence, it is tempting to speculate that innervation might regulate limbal stem cell/progenitor cell populations. Ueno et al. provided additional support for this hypothesis, in assays which showed that denervation depletes stem cell/progenitor compartment in cornea [124]. Among the possible candidates for neural regulation of epithelial cells, the combination of Substance P, which is abundant at corneal innervation [125–127] and stimulates cell proliferation [128], with IGF-I promotes migration [129]. In the next future, research should pay attention on the signals that mediate the interaction of nerves at the limbal crypt.

3.2 *Corneal Epithelial Stem Cells and Their Niche: Basal Membrane and Extracellular Matrix Components*

Since tissues with unique cellular propertiesmay synthesize different substrates to which the cells adhere, authors carried out the biochemical and immunological characterization of the Extracellular Matrix (ECM) components associated with corneal tissue. Before the description of the limbus as the possible location of corneal stem cells, it was known that corneal ECM constituents changed during development until adulthood in chick, mouse, bovine and human corneas. Authors described that corneas contained collagen types I–VI [130–133], glycosaminoglycans such as heparan, chondroitin, dermatan and keratan sulfates [134–138], fibronectin and laminin [139], and hyaluronic acid [140]. These initial evaluations also showed that limbal epithelial cells adhere to a more roughed surface, with a more complex arrangement of anchoring fibrils than the one observed in central cornea [141]. This suggested that limbal cells had a different adhesion capacity in comparison with the rest of the epithelium; a fact supported by the larger hemidesmosomal area detected in central corneal cells [141], which could also suggest differences in cell motility between both corneal regions.

To further understand the functional differences between the cornea and the limbus, and therefore, the interaction between epithelial cells and the niche, several authors made a careful analysis of the corneal basement membrane components. These studies lead first to recognize that the composition of basal membrane (BM) between conjunctival, limbal and corneal epithelia is heterogeneous [142]. An additional characterization of corneal BM provided controversial results, given that some authors reported that central cornea BM lacks of collagen IV [143], while others reported that collagen IV was found in both limbus and central cornea [142]. Such disagreement was later explained as a consequence of the shift in collagen IV chain isoforms between the limbus and the conjunctiva [144, 145]; collagen IV α1 (IV) and α2(IV) chains showed a more intense staining at the corneo-limbal border, whereas α3(IV) chain underwent an abrupt reduction at limbus [145, 146]. In contrast, collagen types IV (α3-α4 chains) and XII were present in central cornea [146], although collagen IV (α4 chain) was weakly expressed in such region [145, 147].

The differential composition of limbal BM was extended to other components. It was found that α2-α5, β1-β3, γ1-γ3 laminin chains, as well as nidogen-1 and -2, and agrin, were preferentially expressed in limbal BM [146]. In particular, limbal BM shows patches of components such as agrin, SPARC/BM-40, tenascin-C, laminin γ3 chain and versican, which co-localize with ABCG2/p63/K19-positive and K3/Cx43/desmoglein/integrin-α2-negative cell clusters, assumed to be formed by stem and early progenitor cells [147, 148]. On the other hand, researchers described that BM components such as type XVI collagen, fibulin-2, tenascin-C/R, vitronectin, bamacan, chondroitin sulfate, and versican, co-localized with vimentin-positive cell clusters containing putative late progenitor cells [144–146] at the corneal-limbal transition zone. In contrast, type V collagen, fibrillin-1 and -2,

thrombospondin-1, and endostatin were almost restricted to the corneal BM [145]; while others, such as type IV collagen α5 and α6 chains, collagen types VII, XV, XVII, and XVIII, laminin-111, laminin-332, laminin chains α3, β3, and γ2, fibronectin, matrilin-2 and -4, and perlecan, were uniformly expressed throughout all ocular surface epithelia [145, 146].

Together, these results suggest that BM at the LEC/LC has a specific ECM composition, different to that found in peripheral and central cornea; probably creating a specialized environment that regulates stem cells and their progeny. Such environment should support stemness, by inhibiting the expression of the differentiation process and preserving the proliferative abilities of limbal cells.

3.3 Corneal Epithelial Stem Cells and Their Niche: Growth Factors and Cytokines

Growth factors have an important role in epithelial maintenance and wound healing. Their role on corneal epithelial cell proliferation and regeneration has been studied either by in vivo assays [148–150], organ culture [148], or by cell culture [151–153].

Although different researchers have described the effect of different growth factors on corneal epithelial cells, the accumulated evidence suggests that Epidermal Growth Factor (EGF) [148, 151, 154–156] and Fibroblast Growth Factor (FGF) [149, 151, 153, 156] are the main proliferative and migratory regulators for corneal epithelial cells, as also seen in epidermal keratinocytes [157–161]. These in vitro results are supported by: (i) The immunolocalization of EGF receptors and aFGF protein at the corneal epithelium [162, 163]; (ii) experiments which show that the corneal epithelial basement membrane possesses a high capacity to bind FGF [164, 165, 255]; and (iii) in vivo assays that demonstrate the stimulation and improvement of ocular surface wound healing by EGF [165–168] or FGF [153, 169].

In spite of the abundant literature describing the effect of growth factors on corneal epithelial cell proliferation and migration, there are few studies dedicated to understand the regulation of limbal cell populations by these molecules. One of the earliest reports suggesting a differential susceptibility of basal limbal cells to growth factors showed that there are higher levels of EGF receptor in basal limbus than in basal central corneal cells mainly during early development [162]. Later, Tseng and collaborators found that bFGF, TGF-α/EGF receptor, IL-1 beta/IL1-receptor, and bFGF/FGF receptor-1 were more expressed by corneal than limbal epithelial cells [170]. These studies suggested that limbal cells have a different regulation, probably related with their less frequent progression through the cell cycle [43].

Further analyses suggested that regulation of limbal stem cells and progenitor cells involves genes that encode proteins that participate in signaling pathways, which control cell cycling and self-renewal such as WNT and Notch [171, 172]. These results also increase the interest in cell-cell interactions that implicate the

asymmetric signaling between neighboring cells [172], and the activation of genes transcribed by proteins activated by β-catenin [61]. Therefore, it was shown that Wnt/β-catenin signaling increases the proliferation and colony-forming efficiency of primary human LSCs, and at the same time preserves the high expression levels of putative corneal epithelial stem cell markers, and low expression of terminal differentiation markers [61]. On the other hand, the down regulation of Notch correlates with an increase in cell proliferation [172].

Thus far, the role of growth factors and cytokines as regulators of stem cells at the limbal niche is poorly understood. This is a consequence of the intricate network of signals that participate in stem cell regulation, as well as the result of the lack of adequate methods and molecular markers useful for stem cell isolation/purification. However, a more extensive discussion of the activities of the limbal niche components will be addressed in the next sections.

4 The Niche as Regulator of Limbal Stem Cells

Considering thedifferential composition between limbal and central corneal basement membranes, as well as the differential responsiveness of the limbal stem cells in relation to central corneal basal cells, it is clear that microenvironment has a tremendous, dramatic effect on corneal epithelial stem cells. The evidence that supports the role of the niche and provides the best examples of the influence of environment on epithelial differentiation was obtained from recombination experiments. In these studies, murine vibrissae hair follicle stem cells were induced to differentiate into corneal epithelial cells by cultivation in a limbus-specific like microenvironment [173]. Under such conditions that comprise laminin-5 as a major component and the addition of medium conditioned by limbal stromal fibroblasts, cells isolated from hair follicles formed stratified epithelia that expressed corneal-specific markers such as K12 keratin and transcription factor Pax6, both at mRNA and protein level, while showing a strong down-regulation of the epidermal specific K10 keratin [173]. Alternatively, in other experiments, central corneal epithelial cells from adult rabbit were recombined with mouse embryonic dermis, leading to the loss of the corneal-specific phenotype and a down-regulation of Pax6. The loss of expression of the corneal-specific K3/K12 keratin pair was accompanied by the induction of basal keratinocyte markers such as the K5/K14 keratins and the differentiation into epidermal keratinocytes, including cells with a hair follicle lineage phenotype [174]. Altogether, these experiments emphasize the effects of microenvironment on the programming of epithelial cells into specific lineages. Since cell fate may be regulated by specific signals arising from the basement membrane, including growth factors and cytokines, it is possible that in the cornea, the decision to leave the stem cell compartment could be dependent on ECM composition and structure at the limbus.

Under such circumstances, corneal epithelial stem cells could follow one of two alternative, different courses. The first establishes that stem cells and their progeny

proliferate by means of horizontal, symmetric divisions. This proliferative pattern would be prevalent at the basal layer of the cornea, including limbus, while stratification and expression of terminal phenotype would depend upon vertical asymmetric cell divisions. Such asymmetric divisions would result in daughter cells dissimilar in both morphology and proliferative potential; as a result of the division, those cells that enter into the suprabasal compartment would be bigger and suffer a severe restriction in their proliferative abilities to begin terminal differentiation [175, 176] (see Fig. 3). In this case, ECM would modulate proliferative abilities of basal cells according to their position along corneal surface (limbus vs. central cornea); and would control the orientation of the mitotic spindle, being decisive for terminal differentiation. So, cells that detach from the basement membrane would be irreversibly committed to express a differentiated phenotype. The possible participation of vertical asymmetric division as a mechanism to establish the expression of terminal phenotype is supported by the observation that most basal cells in the corneal epithelium express proteins involved in spindle orientation, such as Partner of inscuteable (Pins) [177].

In the other, alternative pathway, asymmetric cell division is restricted to the limbal stem cells, as proposed for most stem cells [178]. If this is true, the decision to leave the stem cell compartment would depend upon asymmetric divisions, that would be oriented either horizontally or vertically (Fig. 4). Consequently, symmetric cell divisions would be merely proliferative, and would not be essential for cell commitment. Consequently, the orientation of the mitotic spindle during asymmetric cell division would be defined by extrinsic mechanisms, i.e. the niche or microenvironment in which stem cells reside [179] (Fig. 4). To support this proposal, there are numerous BM components [141, 142, 144, 147, 180], as well as growth factors and cytokines such as keratinocyte growth factor (KGF) [181], IL-6 [182], EGF and, FGFβ [183], or molecules belonging to the Wnt family [61], among others, which show a differential composition or distribution at limbal, peripheral and central cornea. Together, they may be involved in the establishment of the corneal niche.

According to the second model, stem cells at the limbus undergo either vertical or horizontal asymmetric mitosis during corneal replenishment or during wound healing. After asymmetric cell division, one of the daughter cells loses contact with the limbal BM either by moving into the suprabasal cell layers or by moving and proliferating into the central cornea, and initiates the differentiation process [179] (Fig. 4). When such an event occurs, daughter cells also become regulated by the components of the central cornea basement membrane and growth factors such as IGF-I [183], or molecules of the Wnt family as Wnt3, Wnt7a, Wnt7b, and Wnt10a, which are up regulated in central cornea and limbus [61, 184, 185].

Bearing in mind the differences in basement membrane composition between limbus and central cornea, it is quite possible that the differential distribution of ECM components [144–147] regulates limbal epithelial stem cell character. This is supported by results that demonstrate the influence of ECM parameters such as stiffness and elasticity, on the differentiation, proliferative and migratory abilities of embryonic, mesenchymal, or adipose-derived stem cells [186–188].

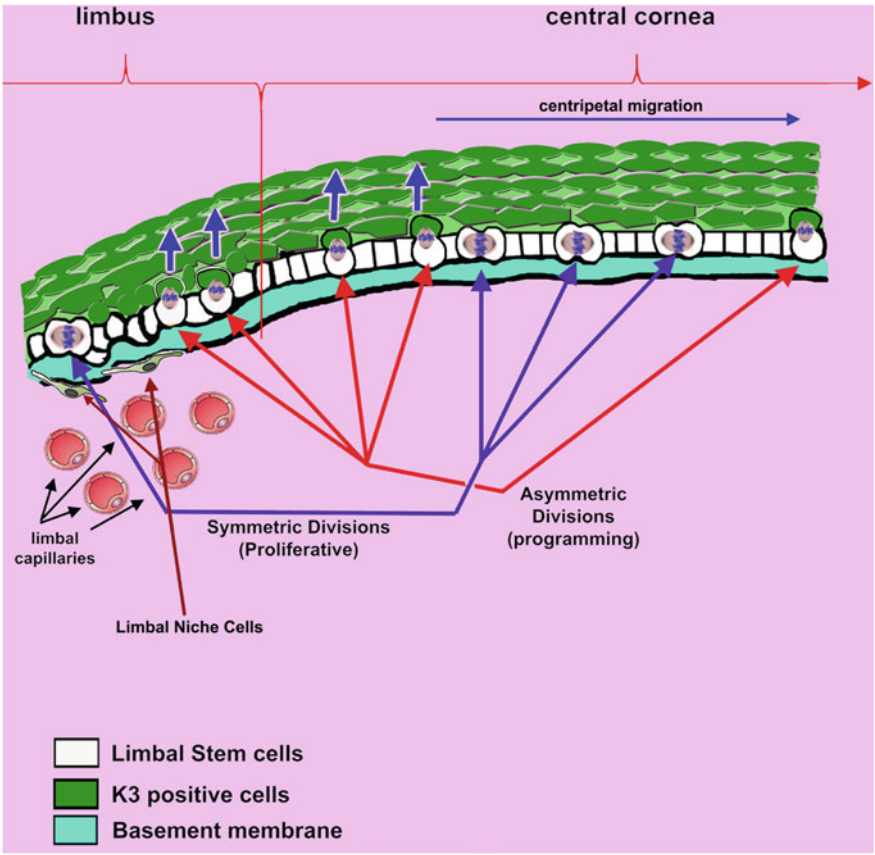

Fig. 3 Representation of corneal epithelial cell renewal dependent on proliferative symmetric mitosis of stem cells and their progeny. Stem cells and their progeny proliferate by means of horizontal, symmetric mitosis; on the contrary, asymmetric cell division only occurs in those cells that start stratification and the expression of terminal phenotype. In such case, basal cells that initiate the expression of terminal phenotype divide with a vertically oriented mitotic spindle; one of the daughter cells remains at the epithelial basal cell layer maintaining its proliferative abilities, and the other leaves the basal layer entering into the suprabasal compartment, becoming bigger and losing its proliferative abilities becoming terminally differentiated (*pink* cells). On this model, detachment from basement membrane (BM) would determine the programming of basal cells into terminal phenotype expression, modulating self-renewal and proliferative abilities of stem cells and their progeny on basis of its composition and structure. *Green* limbal BM. *Orange* peripheral and central cornea BM. *Yellow* conjunctival BM. *Blue arrows* stratification of terminally differentiating cells

Nevertheless, currently there is a debate about the role of stem cells regarding their interaction with the niche. Are they passive entities that respond to systemic or tissue signals by merely adapting their activity to tissue demands? Alternatively, do stem cells affect the surrounding tissue, having a more direct activity on the niche

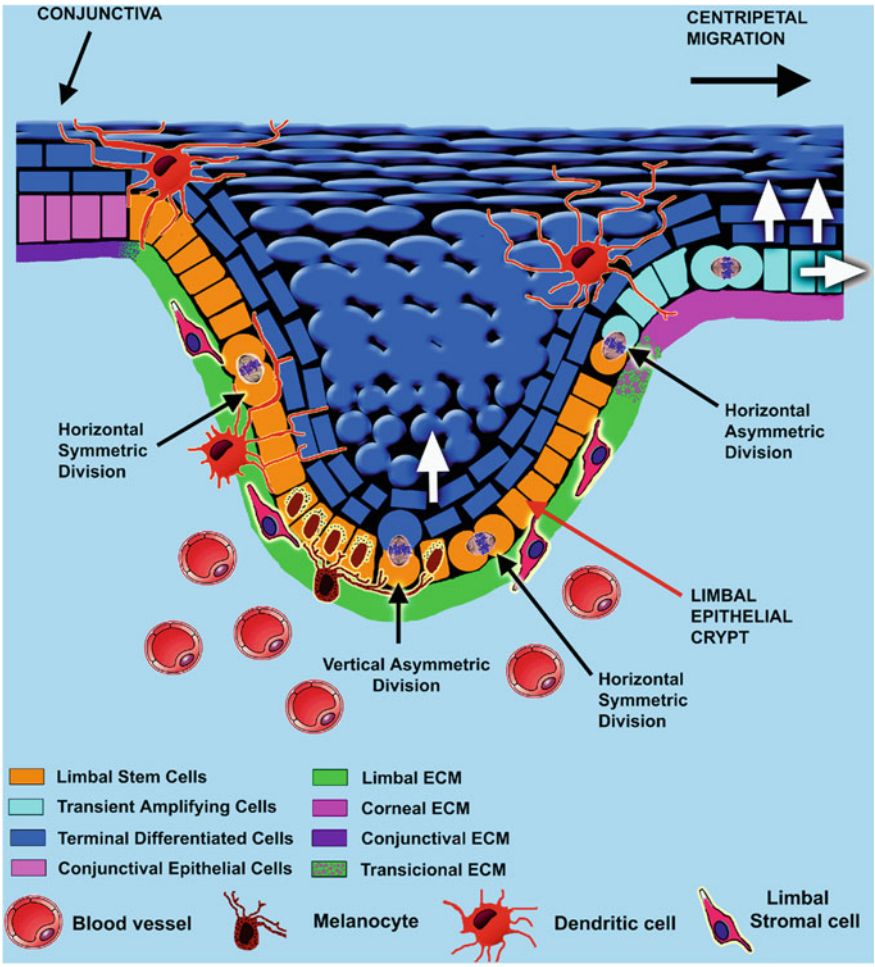

Fig. 4 Schematic representation of the limbal epithelial crypt. Here is shown the complex interrelationships between limbal epithelial cells with different factors that participate in the establishment of the niche. Accumulated evidence suggests that extracellular matrix composition and structure may regulate limbal stem cell fate providing information about their position. Depending on the position of cells at the limbal epithelial crypt, the orientation of mitotic axis during asymmetric cell division of limbal stem cells could be either vertical or horizontal. An asymmetrical dividing stem cell would give rise to another stem cell and a transient amplifying basal cell that would migrate to peripheral cornea when division occurs in a horizontal axis. Conversely, the stem cell could give rise to another stem cell and a limbal suprabasal differentiated cell when division takes place following a vertical axis; in such case, loss of contact between one of the daughter cells and basement membrane would determine the initiation of the differentiation process. *White arrows* indicate the movement of cells after commitment. Differentiation leads to the expression of terminal phenotype

where they reside? It is still unknown how limbal stem cells influence the surrounding cells, tissues, and organs, and therefore, the way in which they can modify their niche. Although there is some evidence regarding the participation of the family of Notch receptors and their associated signal transduction pathway in the regulation of corneal stem cells [172, 189–191], more knowledge is needed about how limbal cells interact with the niche to regulate and enhance responses involved in both tissue maintenance and repair.

5 Corneal Epithelial Stem Cells: Renewal and Wound Healing

Beginning with the discoveryof the centripetal cell migration that occurs in the cornea, early studies on epithelial cell renewal led to conclude that the proliferative source of the corneal epithelium resided at its basal cell layer and at the corneal periphery. In such experiments, authors showed that two separate processes participate in the renewal of the corneal epithelial cells: (i) the division of basal cells, mainly at the corneal periphery, with their successive movement into the suprabasal cell compartment; and (ii) the progression of cells across the limbus toward the center of the cornea, before desquamation of superficial cells [36, 192, 193].

Later, as previously discussed, the limbus was proposed as the presumptive location of corneal epithelial stem cells. Accordingly, corneal epithelium consists of a stratified tissue with a high self-renewal rate based on the regenerative capacities of the stem cells located at the basal layer of the limbus and the proliferation of basal cells from the central cornea [40, 43]. In such well-structured tissue, suprabasal cells both at the limbus and at the central cornea undergo terminal differentiation and lose their proliferative abilities. While basal cells located at the central cornea proliferate actively, basal cells at the limbus consist of a mixture of slow-cycling stem cells and cycling transient amplifying cells [43, 103].

As stated by this hypothesis, normal corneal epithelium remains in a steady state in which cell proliferation is necessary only for replacement of those cells lost by terminal differentiation and desquamation. Stem cells located at the LECs divide occasionally [40, 46, 104, 179], and subsequently, their progeny leaves the niche, while undergoes the transient amplification process, which occurs at the basal cell compartment of the peripheral and central cornea [40, 46, 104, 179]. Such transient amplification would imply a gradient or hierarchy of cells with a decreasing proliferative potential along the central cornea [46, 104], and comprises a still unknown number of cell divisions, mainly modulated by growth factors and cytokines [194–196] before cells become post-mitotic and begin to stratify.

This hypothesis recently received support from mosaic analysis of eyes in mice [256, 197, 198], from lineage tracing on eye surface [198, 199], and from chromosome in situ hybridization on human tissue [200]. These experiments documented centripetal movement of cells from limbus to central cornea. Moreover,

after a wound damage, trauma or exposure to tumor promoters such as TPA, the tissue's response consists of a rapid 8-9-fold rise in the proliferative activity at the limbus, which then is reduced to pre-trauma levels after 36–48 h, as well of a prolonged 2-fold increase in proliferation at peripheral/central cornea which returns to basal levels after wound closure [43, 47]. These results have been interpreted as a consequence of the recruitment and multiplication of the limbal stem cells, and the transient multiplication of the peripheral and central cornea basal cells, respectively [104, 201], and show that limbus contributes to corneal renewal while corneal progenitor cells possess the ability to maintain the corneal epithelium for several months [198, 199].

This possibility is supported by several lines of evidence which suggest that corneal stem cells reside at the limbus, mainly: (i) the lack of an adequate healing of wounds in corneas in which the limbus has been damaged or surgically removed [202–204], (ii) limbal transplantation to restore wound repair [205], or (iii) the presence of holoclone-forming cells in limbus but not in central cornea [31, 46], among others.

So, the reader may ask, which is the role of the niche in corneal wound healing? The answer is mostly unexplored. However, results from different groups suggest that niche rules stem cell behavior through regulation of cell division pattern, in part through an active role of basement membrane components at the limbus. Recent results strongly support the fact that in adult corneal epithelium asymmetrical divisions may only occur at the limbus [179], together with evidence that restricts the expression of specific markers and the expression of cell proliferation and cell fate regulators such as ΔNp63α [59] and Notch1 [206] to stem cells, suggest that asymmetrical cell division is part of the differentiation program in corneal epithelial cells [207]. Therefore, basement membrane would provide limbal stem cells with information about their position and fate. Hence, depending on the position of cells at the limbal epithelial crypt, the orientation of the mitotic axis during asymmetric cell division of limbal stem cells could be either vertical or horizontal. Consequently, an asymmetrical dividing stem cell would give rise to another stem cell and either a transient amplifying basal cell located at the peripheral cornea (when the division occurs in a horizontal axis), or a limbal suprabasal differentiated cell (when the division takes place following a vertical axis).

Accordingly, corneal wound healing should elicit a tissue response in which limbal stem cells undergo few cell cycles and give rise to numerous transient amplifying cells that constitute the migratory/proliferative edge of the wound. The size of the transient amplification of early precursors and committed cells, would then be modulated by changes in the ECM composition and its ECM receptors during corneal wound healing [208–210], and by changes in the expression of growth factors such as IGF-1 [183], Epiregulin [211] or Stem Cell Factor (c-kit ligand) [212].

6 Limbal Stem Cells and Therapy

The current expansion in research on the possible therapeutic use of stem cells also had an impact on the analysis of limbal stem cells and corneal epithelial differentiation. Most authors have approached the use of either limbal stem cells or embryonic stem cells to generate devices for the treatment of corneal damage associated to external agents such as burn injuries, or limbal stem cell deficiencies related with diverse pathologies and hereditary diseases [reviewed in 213].

Among the different strategies utilized by different groups, the transplantation of limbal epithelia whether surgically obtained from the contralateral limbus [205, 214], from donor tissue [215, 216], or bioengineered epithelia [217–220] are, by now, the most valuable tools for corneal surface reconstruction and the relief and cure of limbal stem cell deficiency (LSCD). Independently from variability in patient selection, the type of culture techniques, source of donor tissue, biocompatibility of materials and surgical technique, reconstruction of the corneal surface has shown a clinical success of about 60–80 % [214, 221–224]. In most cases, the outcome seems to be related to the presence of limbal stem cells in the tissue used as source for the manufacturing of the grafts, and supports the benefit of the use of limbal stem cells in ophthalmic therapy.

In view of the successful use of limbal stem cells for ocular surface reconstruction, different groups around the world focused on the use of other stem cell sources to engineer corneal substitutes useful to replace damaged tissue. Among these methods, it is important to emphasize on the generation of autologous corneal constructs by cultivation of hair follicle-derived holoclone-forming cells onto a fibrin carrier [225], as well as the use of human oral epithelia [218, 226, 227], mesenchymal stem cells [228, 229], or embryonic stem cells [230].

Based on the formation of multilayered epithelia expressing corneal differentiation markers [218, 220, 225] and its successful clinical application [231, 232], the use of stem cells different from limbal stem cells shows a high potential for corneal reconstruction. However, because stem cell markers persist in the transient amplifying cell population and in the early differentiating cells [105, 106], surgeons are not certain about the cell types transplanted onto the patients. Such unpredictability may lead to long-term graft failure [233].

Alternatively, the study of stem cell regulation by the niche may lead to develop therapies based on the interference or stimulation of the signaling pathways and microenvironmental components that control limbal stem cells. Since growth factors and ECM components regulate migration and proliferation of the transient amplifying cells, with the preceding proliferation of limbal stem cells, it is possible that growth factors and ECM can be used alone or combined, in order to accelerate and improve repair of corneal wounds, and reduce consequences associated with corneal damage. Examples of this approach consist in the application of growth factors to promote corneal wound healing such as EGF [234, 235], basic FGF [236], TNFα and Interleukin-1 [237]; or ECM components as Decorin [238].

Although some results have suggested that treatment of corneal wounds with growth factors or ECM components offers new opportunities for therapeutic intervention, accumulated evidence implies the need of a complex set of growth factors and ECM components, perhaps in a specific three-dimensional arrangement, to improve and accelerate corneal wound healing. This possibility is supported by the application of cultured epidermal sheets as temporary wound coverings on experimental excimer laser corneal ablations. These epidermal sheets increase at about 60 % the reepithelialization rate of wounds, besides reducing inflammation and scarring at the wound site [239]. Such corneal healing improvement has been explained through the synthesis and release of growth factors, cytokines and ECM onto the wound bed by the cultured epidermal sheets [240]. A similar mechanism for enhancement of wound healing could be occurring during treatment of corneal wounds with amniotic membranes [241].

7 Conclusion

So far, the study of limbal stem cells and their regulation by environmental signals, either cytokines, growth factors, and their interaction with other cell populations is almost unexplored. As mentioned, corneal epithelial stem cell niche constitutes one of the best examples in which niche can be envisioned as the result of a complex mixture of variables that interact and establish the microenvironment to enable the maintenance of stemness and the renewal and repair of the corneal epithelium. So far, we still ignore whether epithelial stem cells exert a reciprocal effect on the niche, although the existing indications do not support this possibility.

Despite researchers have identified a set of molecular markers that may be used for enrichment of stem cells in isolated populations, the results led to conclude that there is not a specific, unique marker for identification and isolation of limbal stem cells [171, 242–244]. The lack of such markers has become one major obstacle to develop therapies based on cell transplantation. Nevertheless, this collection of markers allowed the characterization of the stem cell niche, and demonstrated that the limbus shows special characteristics, both in composition and/or structure, which make it different from peripheral and central cornea [244].

The above evidence, together with cell culture and clonal assays, suggests that the corneal epithelial cells comprise two different populations: stem cells and transient amplifying cells. The latter corresponds to the progeny of the stem cells, and possesses a limited proliferative potential and it is probably committed to terminal differentiation. The number of cell cycles undergone by transient amplifying cells depends on stimuli from the environment [104, 194, 245].

Although numerous studies indicate that corneal epithelial stem cells reside preferentially at the basal layer of the limbal zone rather than uniformly in the entire corneal epithelium, recent results suggest that corneal stem cells may also be at the central cornea [246]. Moreover, other results suggest that corneal wound healing does not necessarily depend on limbal cells [247]. In spite of the controversial

nature of these results, they bring up many questions about the possible function of corneal stem cells during tissue renewal or their migratory potential from the limbus. In either of these cases, a major question involves the possible conditioning effect of stem cells upon environment: Can stem cells modify their surroundings in order to form new niches? The possible location of epithelial stem cells in the central cornea could help to explain the transdifferentiation of adult corneal epithelium when it receives signals from embryonic dermis [174], unless researchers could demonstrate that expression of corneal epithelial phenotype is reversible by stimulation of the appropriate signaling pathways.

Understanding of the niche'sbiological activity on stem cells, may lead us to develop new therapies to accelerate and improve corneal wound healing.

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