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REVIEW

Niche regulation of corneal epithelial stem cells at the limbus

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Among all adult somatic stem cells, those of the corneal epithelium are unique in their exclusive location in a defined limbal structure termed Palisades of Vogt. As a result, surgical engraftment of limbal epithelial stem cells with or without *ex vivo* expansion has long been practiced to restore sights in patients inflicted with limbal stem cell deficiency. Nevertheless, compared to other stem cell examples, relatively little is known about the limbal niche, which is believed to play a pivotal role in regulating self-renewal and fate decision of limbal epithelial stem cells. This review summarizes relevant literature and formulates several key questions to guide future research into better understanding of the pathogenesis of limbal stem cell deficiency and further improvement of the tissue engineering of the corneal epithelium by focusing on the limbal niche.

Introduction

Unlike the rest of the body surface, the ocular surface is designed to be wettable so as to maintain comfort while providing a smooth optical surface. On the ocular surface, the cornea is most unique in being both avascular and transparent so as to allow the light to be transmitted to the retina. Therefore, in order for us to enjoy seeing the outside beautiful world, the corneal epithelium needs to withstand constant attrition caused by exposure-induced dryness and potential light-induced damage.

To cope with the aforementioned demand, one fundamental strategy resorts to prolonged, if not indefinite, self-renewal of the corneal epithelium. This unique property is governed by corneal epithelial stem cells (SCs), of which the location was discovered by the laboratory of Dr Tung-Tien Sun 20 years ago to be exclusively in the basal layer of the limbus, i.e., the outer vascular rim at the junction between the cornea and the conjunctiva [1]. This important discovery has helped resolve the mystery of "conjunctival

transdifferentiation" thought to take place in the event of a total corneal epithelial loss [2], explained why a number of corneal blinding diseases manifest limbal SC deficiency with cytological evidence of conjunctivalization [3], and devised a new surgical procedure of transplanting the SC-containing limbal epithelium for treating eyes inflicted with limbal SC deficiency [4].

A number of studies have since disclosed that limbal SCs share some features common to other adult somatic SCs. For example, limbal SCs have the smallest cell size [5], are slow-cycling and hence label-retaining [6], and do not express markers destined for terminal differentiation such as cytokeratins 3 [1] and 12 [7-9], involucrin [10], and connexin 43 [11]. In contrast, the SC-containing limbal epithelium has a high proliferative potential in different cultures [12-15], and their *in vitro* proliferation is resistant to the inhibition by tumor-promoting phorbol esters [13, 16, 17]. Furthermore, limbal basal epithelial cells express cytokeratin 19 [18], and integrin α9 [10, 19], and preferentially express such progenitor markers as p63 [20], especially its ΔNp63α isoform [21, 22], Bcrp1/ABCG2 [10, 23-25], and N-cadherin [26].

Compared to other types of adult somatic SCs, limbal epithelial SCs are unique in being enriched in an anatomic location that is relatively easy to access, hence rendering it an attractive model to investigate the underlying regula-

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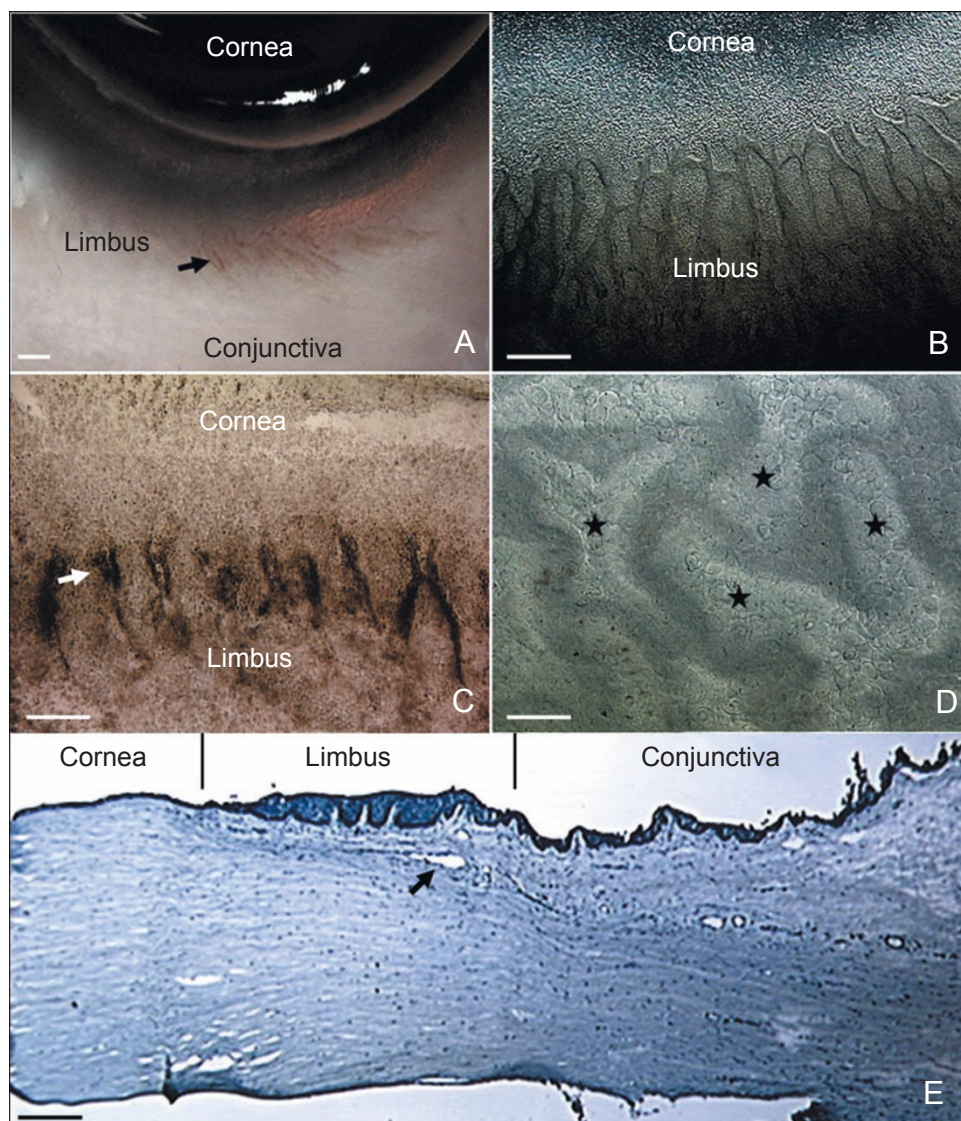E-mail: stseng@ocularsurface.com

Figure 1 The limbal palisades of Vogt. Palisades of Vogt (arrow) are readily recognized in the human limbus **(A)**. Such a unique pigmented structure can be identified on the flat mount preparation of Dispase-isolated human limbal epithelial sheets **(B)**. In donors with a darker skin, these palisades of Vogt are pigmented (**C**, arrow). Under higher magnification, these limbal areas show undulated epithelial papillae (**D**, stars). Hematoxyline staining highlights higher stratification and more undulation of the limbal epithelium, and the underlying limbal stroma has high cellularity and vascularity (E, arrow shows blood vessel). (Bar represents 500 μm in **A** and **B**, 200 μm in **C** and **E**, and 50 μm in **D**) (A, B, [127])

tory mechanism. The same reason has made it possible to perform several surgical procedures to engraft both autologous and allogeneic limbal epithelial SCs to restore sight in animal [27-29] and human [4, 30-32] limbal-deficient corneas. Furthermore, several new surgical approaches based on transplantation of *ex vivo* expanded limbal epithelial SCs have also been attempted [33, 34]. These new advances collectively make the corneal/limbal epithelia a prime tissue to practice regenerative medicine. For more

detailed information on the aforementioned progresses regarding limbal epithelial SCs, the reader is encouraged to consult several reviews [35-45]. Further understanding of how self-renewal and fate decision of limbal epithelial SCs are regulated will undoubtedly unravel their additional therapeutic potentials in the future.

Increasing evidence supports that adult germ and somatic SCs are regulated by their niche, i.e., a special microenvironment consisting of other cellular and extracellular
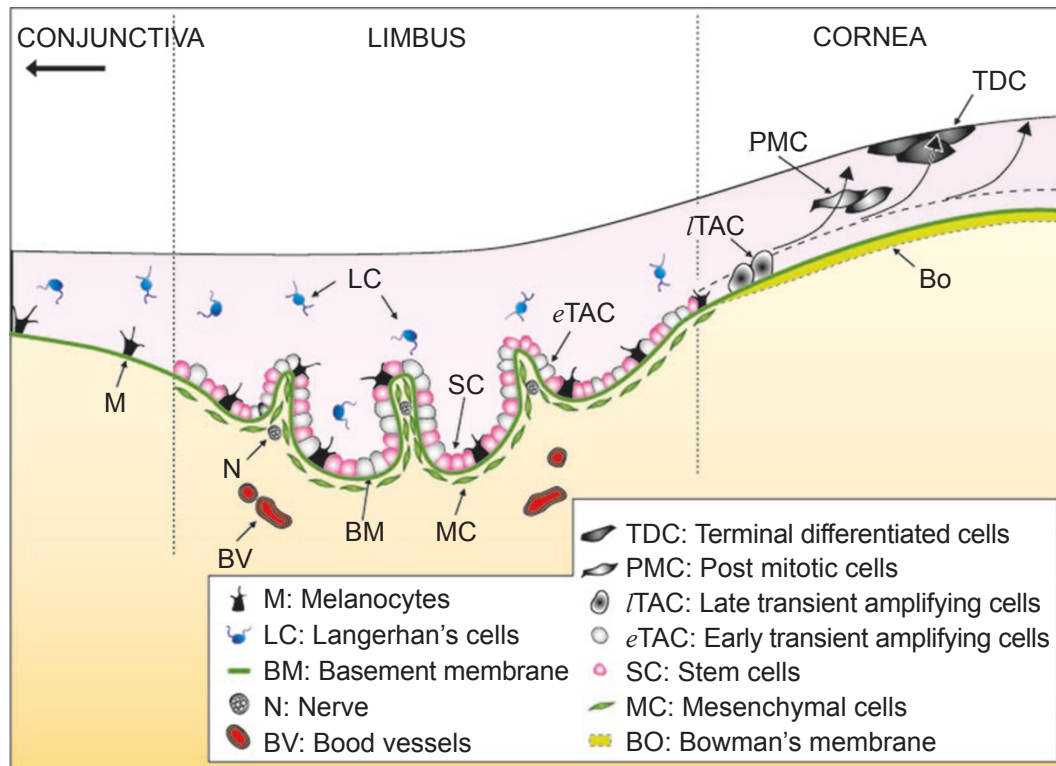
Figure 2 Hypothetical scheme of limbal stem cell niche. Limbal epithelial stem cells (SC) are located at the limbal basal layer. In this epithelial level, there are several other cell types in the vicinity such as the immediate progeny, i.e., early transient amplifying cells (*e*TAC), melanocytes (M), and Langerhan's cells (LC). It remains to be determined whether these cell types act as niche cells. It is believed that eTAC will be destined for progeny production by differentiating into late TACs (*l*TAC) located at the corneal basal layer, then into suprabasal post-mitotic cells (PMC), and finally into superficial terminally differentiated cells (TDC). The limbal basement membrane (BM) separating the epithelium from the underlying stroma has several unique components. The subjacent limbal stroma contains mesenchymal cells (MC), which may also serve as niche cells. Because the limbal stroma is highly innervated and vascularized, the respective role of nerves (N) and blood vessels (BV) in the niche remains to be defined.

components in the vicinity (for reviews see [46-48]). Therefore, similar to what has been carried out in other types of SCs, one important way of exploring the biological regulation of limbal SCs is to understand how they are regulated by their niche. This review intends to summarize the current status regarding how much we know about the limbal niche. We would like to raise several key questions that may fill in the missing gaps regarding how the limbal niche might regulate limbal epithleial SC's self-renewal and fate decision. Under each question, we also discuss areas that await future research.

Where is the limbal stem cell niche?

The SC niche has both anatomic and functional dimensions. Before functional dimension can be addressed, it is necessary to understand where the limbal niche is. Ana-tomically, the limbal SC niche is located at the Palisades of Vogt (Figure 1), which is highly pigmented because of melanocytes [49-51], and is infiltrated with antigen-present-ing Langerhan's cells [52] and suppressor T-lymphocytes [53]. Unlike that of the cornea, the basement membrane of the limbus is undulating with papillae or 'pegs' of stroma extending upward [54] and fenestrated [55, 56]; these ana-tomic features in the limbus suggest that limbal epithelial SCs might closely interact with cells in the underlying limbal stroma (Figure 2). The preferential expression of α9 integrin [19] and N-cadherin [26] without connexin 43 [11] also suggests that limbal SCs interact with unique extracellular components in the niche. Compositionally, other than laminin-1 and laminin-5, the limbal basement membrane also expresses laminin α2β2 chains, while the corneal basement membrane does not. Moreover, α1, α2, and α5 chains of type IV collagen are present in the limbal

basement membrane, while α3 and α5 chains are in the corneal counterpart [57, 58]. These limbal basement membrane components might help determine SC distribution in the niche as suggested in the intestinal crypt villus (for review, see [59]). Furthermore, like that of other SC niches [60, 61], the limbal basement membrane might help sequester and hence modulate concentrations of growth factors and cytokines that are released from limbal niche cells for efficient and precise targeting onto limbal SCs.

Underneath the basement membrane, the limbal stroma is heavily innervated [62] and vascularized [63] (Figure 1). Cells residing in the limbal stroma are heterogenous and yet poorly defined. Besides resident mesenchymal cells, one cannot ignore the contribution of bone marrow-derived cells migrating into the limbal stroma [64]. The physical closeness between limbal epithelial SCs and their underlying or surrounding "niche cells" has not been demonstrated, nor has the dependence of limbal SC functions on such close interactions.

By definition, niche cells provide a sheltering environment that shields SCs from stimuli that may adversely promote differentiation and apoptosis, threatening SC reserves [47]. In this regard, melanocytes, distributed in the limbal basal layer with their cellular projections extending to surrounding basal epithelial cells [51], may be one candidate of niche cells (Figure 2). These cells produce and transport melanin pigments into epithelial cells so as to minimize damage caused by ultraviolet irradiation, a presumed action similarly described in the SC-containing bulge area of the human skin [65]. Little is known about the molecular mechanism explaining cytokine dialogues operating between melanocytes and limbal SCs, let alone between those of other cells in the vicinity.

Is there any evidence suggesting niche regulation of limbal epithelial SCs?

It is generally accepted that the differentiation of lineage-committed SCs into mature progenies is a one-way biological process. When rabbit limbocorneal explants were cultivated at an air-fluid interface, epithelial proliferation and stratification is promoted [66]. In this model, intrastromal invasion of basal progenitor cells occurs only in the limbal, but not the corneal region [67]. Such intrastromal invasion of limbal basal epithelial progenitor cells only occurs when the limbal epithelial tissue is recombined with the live, but not devitalized, limbal stroma, and is abolished when recombined with the live corneal stroma [67]. When the corneal epithelial tissue is recombined with the live limbal stroma, there is no intrastromal invasion [67] whereas the basal epithelial cells lose expression of cytokeratin 3 and connexin 43, indicative of de-differentiation of corneal

transient amplifying cells (TACs) [66]. Conversely, when the limbal epithelial tissue is recombined with the live corneal stroma, basal epithelial cells express cytokeratin 3 and connexin 43, indicative of terminal differentiation [66]. Collectively, these findings strengthen the notion that the limbal stromal microenvironment indeed plays an important role in down-regulating epithelial differentiation, and that limbal stroma may contain niche cells to promote the SC phenotype. Furthermore, they also indicate that the insult of air-exposure is sufficient to alter the limbal niche to promote intrastromal invasion of limbal basal progenitors via epithelial-mesenchymal transition (EMT) [67]. Future studies of the underlying molecular mechanism may help unravel whether and how limbal mesenchymal cells may serve as potential niche cells to modulate limbal SCs.

Remarkably, well-differentiated adult corneal TACs can also be reprogrammed to de-differentiate into epidermal tissues in response to embryonic dermal stimuli [68, 69]. Such trans-differentiation occurs in a multiple-step process first involving translocation of PAX6 from the corneal epithelial nucleus to the cytoplasm and then its complete repression [70]. Subsequently, some critical signals in the Wnt pathway including β-catenin and Lef-1 are upregulated. Finally, the corneal cytokeratin pair 12 and 3 is replaced by skin cytokeratins 5, 14, and 17. The development of pilosebaceous and sweat glands further implies that corneal-derived hair follicles contain a source of multipotent cells similar to those usually found in the bulge region of the skin. The fact that the embryonic dermal niche can turn corneal TACs into multipotent skin precursors provides strong evidence supporting the notion that the underlying stromal niche of each tissue is unique and is a potent modulator of the plasticity of not only SCs but also TACs.

How can limbal epithelial SCs and their niche cells be identified?

The first step to study the limbal niche is to identify where limbal epithelial SCs exactly lie before one can trace its vicinity with accuracy. One common method to identify limbal epithelial SCs is to label them according to their slow cell cycle [6]. Using this method, epidermal [71] and cardiac [72] niches have been identified. No study has been conducted in the same manner to identify the cellular components of the limbal niche. The other method to identify limbal epithelial SCs is based on their expressed markers. Although limbal epithelial SCs preferentially express several markers (see above), unfortunately there is no consensus *bona fide* marker [44, 45]. It is highly plausible that limbal epithelial SCs may express a set of genes as their "signature" rather than a single one, similar to what has been found for epidermal keratinocyte SCs [71,

73]. Both the label-retaining pattern and immunostaining patterns of several putative markers have revealed that not all basal cells are SCs (for review see [44]), a finding consistent with the notion that true SCs represent only a small fraction in a given tissue [74]. Taken together, the heterogeneity of limbal basal cells also suggests that limbal SCs are physically in close contact with their immediate progeny, i.e., TACs. It remains to be elucidated whether TACs in the limbus differs from those located in the corneal basal layer based on the stage of differentiation into early vs. late TACs, respectively (Figure 2). It also remains to be determined whether limbal TACs, like those in the cardiac niche [72], may play a role in instructing the SC fate in the niche. Intriguingly, some limbal basal cells also express vimentin [18, 44], a mesenchymal cell marker. Because both Langerhan's cells and melanocytes that reside in the limbal basal layer also express vimentin, future studies are needed to determine whether limbal epithelial SCs also express vimentin or instead these vimentin-expressing cells actually represent niche cells. Clarification of these questions may help identify limbal niche cells and their interactions with limbal epithelial SCs.

How can limbal epithelial SCs and their niche cells be isolated?

Once the limbal niche is identified, the next obvious step is to isolate it from the *in vivo* habitat so that cells in the niche can be further investigated. To this end, intact limbal epithelial sheets can be successfully isolated by digestion with Dispase from several species [66, 75, 76]. Single cells rendered by a brief treatment with trypsin and EDTA of such isolated limbal epithelial sheets have been used by many investigators as the primary source of limbal epithelial SCs for subsequent manipulations including fluorescent-activated cell sorting (FACS) and culturing. It remains unknown whether different protocols of Dispase digestion actually remove the entire limbal epithelial SCs, niche cells or both. This concern is raised because a recent study showed that there are invading 'crypt-like" structures in human limbal palisades of Vogt [55]. Furthermore, this concern is justified because as described above limbal basal progenitor cells can invade into the limbal stroma [67]. If indeed both limbal SCs and their niche cells were completely removed from the *in vivo* habitat by Dispase digestion, it remains unknown whether subsequent trypsin/EDTA treatment might disrupt their intrinsic intercellular connections, and whether such disruption might then affect the success of subsequent cultivation/expansion. If however only limbal SCs were isolated, there has not been any attempt made to isolate niche cells from the remaining limbal stroma.

Based on the principle that many adult somatic SCs preferentially express Bcrp1/ABCG2, a member of ATP-binding cassette transporters, they can be isolated as the side population (SP) using FACS via the unique property of effluxing Hoechst 33342 dye [77, 78] (for review see [79]). Using this method, SP cells have been isolated from human [23-25], rat [80], and rabbit [24, 81, 82] limbal tissues. The frequency of SP cells from the freshly isolated limbal epithelium varied from 0.2% to 0.64% in humans and from 0.4% to 1.21% in rabbits, while no SP cells were detected in human and rabbit central corneas. Interestingly, a recent study showed that 4.6% of SP cells are isolated from the rat central corneal epithelium, which is significantly higher than 0.4% from the rat limbus [80]. Future studies are needed to determine whether ABCG2 is expressed not only in the limbal tissue, whether other members of ABC transporter family may be expressed by some corneal cells, and whether cells other than SCs can also express ABCG2. Although epidermal SP cells have been proven devoid of melanocytes or dendritic cells [83], a preliminary study did show that both human and rabbit limbal SP cells contain non-epithelial cells such as lymphocytes [84], raising the question whether it is valid to use SP as a source of limbal SCs.

Will restoration of niche support be critical for *ex vivo* expansion of limbal epithelial SCs?

In as much as it remains uncertain whether the success of aforementioned isolation might be hampered by intrinsic disruption of intercellular interaction/support between SCs and niche cells, much evidence does suggest that restoration of such support is crucial for *ex vivo* expansion of limbal epithelial SCs. In fact, many types of adult somatic SCs have limited functions when detached from their *in vivo* niche. To circumvent this problem, one common approach is to cultivate them on a feeder layer made primarily of growth-arrested mesenchymal cells as a surrogate niche. For many types of epithelial progenitor cells, *ex vivo* expansion resorts to co-culturing on β-irradiated or mitomycin C-treated murine 3T3 fibroblast feeder layers first pioneered by Rheinwald and Green in 1975 [85].

Interestingly, using the clonal culture system based on 3T3 fibroblast feeder layers, SP cells generate much less colonies than non-SP cells in freshly isolated limbal epithelial cells from both human [24] and rabbit [24, 81, 82]. These results prompt one to suspect whether expression of ABCG2 is a salient feature of limbal SCs as discussed above. Nevertheless, SP cells from freshly isolated rabbit limbal epithelial sheets showed a five-fold increase of colonies after corneal epithelial wounding to activate limbal SCs [82]. Furthermore, SP cells harvested from primary

human limbal epithelial cultures yield more colonies than those of non-SP cells [25]. A similar finding was also noted in cultured epidermal keratinocytes [86]. These results suggest that clonal expansion of "quiescent" limbal SCs is less apt on 3T3 fibroblast feeder layers than "activated" limbal SCs, which generate sufficient numbers of TACs. Alternatively, clonal expansion of limbal SCs may require additional support from TACs that is absent on 3T3 fibroblast feeder layers.

Another method is to use amniotic membrane as an *ex vivo* surrogate to expand limbal epithelial SCs in culture [87-89]. This novel approach was derived from the clinical experiences in transplanting cryopreserved human amniotic membrane, i.e., the innermost layer of the placenta, to facilitate ocular surface reconstruction (for reviews see [90-94]). Many diverse action mechanisms have been ascribed to the amniotic membrane for its supplanting with a new basement membrane and a stroma that can suppress inflammation, scarring and angiogenesis (for review see [95]). Importantly, amniotic membrane transplantation alone is sufficient to restore the normal corneal epithelial phenotype in human corneas with partial limbal SC deficiency [96-98], indicating that amniotic membrane helps expand residual limbal epithelial SCs *in vivo*. The above clinical discovery has led others to successfully transplant such an *ex vivo* expanded human limbal epithelial tissue to treat human corneas with total limbal SC deficiency [34, 99, 100]. Because no murine 3T3 fibroblast feeder layer is needed, human amniotic membrane may serve as an *ex vivo* surrogate niche (for review see [39]). Because the resultant epithelial phenotype is "limbal" when expanded on an intact amniotic membrane, which retains the devitalized amniotic epithelial cells, but is "corneal" when expanded on an epithelially-denuded amniotic membrane [101], we began to explore the idea that human amniotic epithelial cells may serve as a better non-xenogenic surrogate niche to support limbal epithelial SCs [Chen *et al*, manuscript submitted, 2006]. Continuous search for the most ideal candidate of *ex vivo* niche cells is important to allow us to expand limbal epithelial SCs effectively for further manipulations.

The finding that amniotic membrane transplantation can treat partial limbal SC deficiency also indicates that amniotic membrane may not only mobilize limbal SCs from the adjacent normal limbal tissue, but also recruit some multipotent progenitors from such a remote location as the bone marrow. The latter hypothetical scenario, if proven, illustrates an exciting new possibility for the limbal niche in directing trafficking and homing of host circulating SCs, an emerging concept well documented in the hematopoietic niche (for review, see [102]), and the *Drosophila* germline niche [103].

Can damaged limbal niche cause limbal SC deficiency?

As stated above, limbal SC deficiency is commonly manifested in many blinding ocular surface diseases. Besides the hallmark of conjunctivalization, i.e., ingrowth of conjunctival epithelial cells, the limbal-deficient corneas also show chronic inflammation, vascularization and scarring (for reviews see [41, 104]). When the cytological evidence of conjunctivalization is used as a clinical tool for diagnosing limbal SC deficiency [3], these diseases can further be subdivided into two major categories [41]. The first category is characterized by the destructive loss of limbal SCs by chemical/thermal burns, Stevens-Johnson syndrome, multiple surgeries, extensive microbial infection, radiation, and anti-metabolite uses. Intriguingly, diseases in the second category do not have such a destructive loss, and yet with time, also manifest the same phenotype of limbal SC deficiency. One prototypic disease in the second category turns out to be aniridia (due to allele mutation of PAX 6). Heterozygous Pax 6-null mice (small eye) also show the pathologic features of limbal SC deficiency [105]. It has been speculated that the limbal niche is dysfunctional in these mouse limbal deficient corneas (for review see [106]). Investigation into the molecular mechanism of how PAX 6 controls the limbal niche is vital (also see below).

Besides aniridia, other causes in the second category include multiple endocrine deficiencies and diverse diseases affecting the peripheral cornea and the limbus of which the common denominator is chronic inflammation in the limbal stroma. Indeed, intensive inflammatory cell infiltration was found in corneal and limbal pannus specimens from patients with total limbal SC deficiency [107]. Furthermore, chronic inflammation threatens the well-being of transplanted limbal autograft [28]. One pathogenic insight into how inflammation may lead to limbal SC deficiency is learned from a recent study showing how limbal basal epithelial progenitor cells may invade into the limbal stroma through the process of EMT when rabbit limbal explants are cultured at the air-fluid interface [67]. This process has been reproduced in human limbal explants when cultured on intact amniotic membrane, a technique as mentioned above used for *ex vivo* expansion of limbal epithelial SCs [108], disclosing one drawback of this protocol to be progressive decline of limbal epithelial progenitor cells migrating onto the amniotic membrane. Because trans-differentiation of epithelial cells to fibroblasts via EMT has been implicated in the pathogenesis of renal and lung fibrosis (for review see [109]), the above findings also help explain why limbal SCs are lost during fibrosis in limbal SC deficiency. Because the above pathologic process

only occurs in limbal basal epithelial progenitor cells when there is live limbal stroma [67], future studies are needed to determine what kinds of damage to the limbal niche by chronic inflammation might trigger limbal SC deficiency. If proven, besides transplantation of limbal SCs, restoration of a healthy limbal niche would be a new strategy for treating limbal SC deficiency.

When and how does limbal stem cell niche form during morphogenesis?

It is well known that the corneal epithelium is derived from the surface ectoderm while the mesenchymal cells of the anterior eye segment predominately originate from the neural crest. During eye morphogenesis, there is intensive interaction between epithelial and mesenchymal cells to precisely control their proliferation, migration, and differentiation. There are two waves of mesenchymal cell migration involved in the anterior eye development; the first wave cells form the corneal endothelium and stromal keratocytes, and the second differentiates into the trabecular meshwork, the iris stroma and the ciliary body (for review see [110]). Much is to be learned concerning when mesenchymal cells reach the limbal area and how they are destined to form the limbal niche.

Serial morphogenic events in embryogenesis are controlled by different transcription factors. The earliest transcription factor expressed when embryonic SCs are destined to the keratinocyte lineage is p63 [111]. However, the first recognized ectodermal gene expression associated with eye development is PAX 6 [112], which is consistently turned on by the ocular surface epithelium postnatally [113]. In contrast, head surface ectodermal cells lacking PAX 6 expression continue a "default differentiation pathway" to generate the epidermis. Several transcription factors such as MAF [114], FOXE3 [115] and PITX3 [116] might be involved in guiding early differentiation of the ocular mesenchyme because dysfunction of these transcription factors can cause Peter's anomaly, of which some may also manifest limbal SC deficiency, a hallmark found in aniridia.

Nevertheless, the full maturation of the limbal niche might extend into the postnatal life. This notion is suggested in the mouse or rat corneal epithelium where some corneal basal epithelial cells still retain SC function postnatally before SCs become sequestered to the limbal area [74, 117-119]. Therefore, if these findings hold well not just for rodents but also humans, future studies are needed to determine whether postnatal stimuli such as eye opening and exposure to the air via the tear film play a role in facilitating the maturation of the limbal SC niche.

What signaling pathways are involved in the niche control of limbal SCs?

SCs need to be communicating with their own niche to maintain their self-renewal and fate decision in generating the progeny. This SC-niche cross-talk may involve cell-cell contact, cell-matrix contact, as well as paracrine factors and their receptors. Different studies have shown that signals such as the Sonic hedgehog (Shh) pathway, Wnt/β-catenin pathway, Notch pathway, and TGF-β/BMP pathway play important roles in the niche control of different types of SCs (for review, see [48]). However, little is known about the cross-talk between limbal SCs and their niche. Recently, mouse null for expression of the *Dickkopf* (Dkk) family number Dkk2, one of the Wnt pathway inhibitors, was shown to lose the corneal fate decision on the ocular surface to epidermal differentiation [120]. As a result, Wnt/β-catenin pathway is upregulated in the limbal, but not corneal, mesenchyme, indicating that Dkk2 acts by inhibiting Wnt signaling in the limbal stroma [120], providing the first evidence for a limbal mesenchymal (niche) role in limbal SC differentiation during morphogenesis. Because expression of PAX 6 is lost in the Dkk2-null corneal epithelium, it is likely that Dkk2 might be an upstream regulator dictating PAX 6 expression in the ocular surface epithelium. Further investigation into this molecular control may not only unravel how the limbal niche is developed but also shed light on how the limbal niche becomes dysfunctional in aniridia. Conditional inactivation of Notch 1 in adult mice induces hyperplasia and keratinization of the corneal epithelium with activation of β-catenin pathway, mimicking epidermal differentiation [121]. However, it remains unknown whether the limbal niche is altered when epithelial expression of Notch 1 is interfered. Besides dysgenesis of the trabecular meshwork and Schlemm's canal drainage structures, peripheral corneal scarring and vascularization was seen in mice with heterozygous deficiency of BMP4 [122]. It would be interesting to know whether the corneal abnormality is due to dysfunction of limbal niche. Another mesenchymal cell derived mitogen for epithelial cells is keratinocyte growth factor (KGF, or FGF-7). Transgenic mice with epithelial overexpression of human KGF leads to hyperproliferation of embryonic corneal epithelial cells, and their subsequent differentiation into functional lacrimal gland-like tissues without K12 keratin expression [123]. This result suggests that the limbal niche is no longer functional in guiding the corneal epithelial differentiation when these cells overexpress KGF. It remains to be determined whether the effect of this cytokine perturbation on limbal niche control of limbal SC behavior is temporally and spatially dependent.

Can limbal epithelial SCs be multipotent when the niche is modified?

Traditionally, cell commitment has been viewed as a series of irreversible steps, involving an increase of commitment to a particular cell lineage and a loss of the capability to differentiate to all others. Therefore, the lack of such commitment by enhanced SC plasticity may lead to "trans-differentiation" and serves as a mechanism to explain how adult tissue-specific SCs could potentially generate other organs' cells [124].

Because limbal epithelial SCs may undergo EMT to become fibroblasts when the limbal niche is damaged, one may speculate that limbal epithelial SCs are not restricted only to differentiation toward the corneal epithelial lineage. Because plasticity leading to de-differentiation and then transdifferentiation into the epidermis can happen even in corneal TACs when engrafted to the embryonic dermis [69, 70], one may further speculate that limbal epithelial SCs might be multipotent if an appropriate stromal niche is provided. Such a hypothesis is supported by the findings that neural differentiation of the limbal epithelium takes place when heterotopically transplanted into the mouse hippocampus [125], and that ectodermally derived limbal SCs display neuronal electrophysiology and gradually express the neural SC marker nestin when removed from the limbal niche to an *in vitro* environment [126]. Although SC plasticity might be altered by culturing conditions, clarification of whether indeed limbal SCs possess multipotency is an important issue. Answers addressed to the aforementioned questions will also help us enhance limbal SCs' plasticity to reveal their multipotency. If such multipotent SCs can be isolated and the instructive signal from the limbal niche be identified, an entirely different strategy of tissue engineering of the corneal epithelium and other tissues will be at hand in the future.

Acknowledgment

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