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Stem Cells of the Adult Cornea: From Cytometric Markers to Therapeutic Applications

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Abstract

The cornea is a major protective shield of the interior of the eye and represents two thirds of its refractive power. It is made up of three tissue layers that have different developmental origins: the outer, epithelial layer develops from the ectoderm overlying the lens vesicle, whereas the stroma and the endothelium have mesenchymal origin. In the adult organism, the outermost corneal epithelium is the most exposed to environmental damage, and its constant renewal is assured by the epithelial stem cells that reside in the limbus, the circular border of the cornea. Cell turnover in the stromal layer is very slow and the endothelial cells probably do not reproduce in the adult organism. However, recent experimental evidence indicates that stem cells may be found in these layers. Damage to any of the corneal layers leads to loss of transparency and low vision. Corneal limbal stem cell deficiency results in severe ocular surface disease and its treatment by transplantating ex vivo expanded limbal epithelial cells is becoming widely accepted today. Stromal and endothelial stem cells are potential tools of tissue engineering and regenerative therapies of corneal ulcers and endothelial cell loss. In the past few years, intensive research has focused on corneal stem cells aiming to improve the outcomes of the current corneal stem cell transplantation techniques. This review summarizes the current state of knowledge on corneal epithelial, stromal and endothelial stem cells. Special emphasis is placed on the molecular markers that may help to identify these cells, and the recently revealed mechanisms that could maintain their "stemness" or drive their differentiation. The techniques for isolating and culturing/expanding these cells are also described. © 2008 International Society for Advancement of Cytometry

Key terms

cornea; stem cell; limbal epithelial stem cells; mesenchymal stem cells; endothelial stem cells; stem cell marker; stem cell niche; side population; ex vivo expansion; transplantation; C/EBP(delta); Bmi-1; ABCG2; p63

THE cornea is the anterior, transparent part of the eye, a circular window with high refractive power that directs light bundles to the retina. Besides its optical functions it is also a very important protecting shield, defending the interior structures of the eye from damage. To fulfill these tasks, it has to possess a mirror-like smooth surface, transparent and avascular substantia propria as well as enough tensile strength and flexibility to resist mechanical damage and keep proper shape. This highly specialized structure consists of five layers (Fig. 1A). The outer surface is covered by the corneal epithelium, a stratified squamous nonkeratinizing epithelium, which continues into the conjunctival epithelium at the edges of the cornea, called the limbus (Fig. 1B). The second layer is the Bowman's layer. It is the outer, acellular zone of the stroma. The third layer, the corneal stroma, makes up about 80% of the corneal thickness and consists of a densely packed yet transparent connective tissue, the transparency of which is thought to originate from its regularly ordered and equally spaced collagen bundles, produced by the corneal fibrocytes called keratocytes. The next layer is the Descemet's membrane, which is the thick basement membrane of the innermost layer, the endothelium. The endothelium separates the cornea from the aqueous

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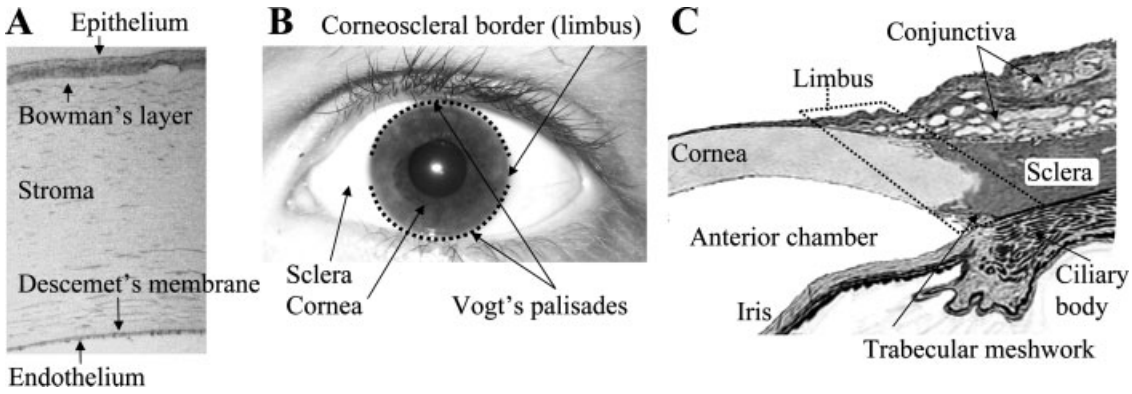

Figure 1. Localization of corneal stem cells. **A**: Histological section and tissue layers of the cornea. **B**: The corneal limbus is localized to the corneoscleral border. The upper and lower regions most protected by the eyelids contain the Vogt's palisades that apparently host most of the corneal epithelial stem cells. **C**: Cross-section of the corneoscleral transition. The corneal epithelium is contiguous with the conjuctiva, the corneal stroma transits into the sclera, whereas the corneal endothelium is linked with the trabecular meshwork. These transitional zones together contain the majority of stem cells in the adult cornea.

humor of the anterior chamber of the eye, provides the stromal keratocytes with nutrients and participates in the maintenance of stromal transparency via its transport functions. Insufficiency of this pump function can result in corneal oedema, which leads to loss of corneal clearness and low vision. The stroma continues into the sclera at the limbus and the endothelium is connected through a so called transition zone with the trabecular meshwork, continuing into the anterior surface of the iris and the suprachoroidal space (Fig. 1C).

At the limbal zone, where corneal tissues give place to other tissues of the eye, corneal structure changes. The epithelium thickens and forms epithelial pegs made up of 10–12 cell layers instead of the five layers observed in the central cornea. The Bowman's layer is missing and the undulated epithelial basement membrane lies directly above the limbal stroma, in which the collagen bundles become less organized and cells are abundant and fibroblast like. In the beginning of the 19th century, ophthalmologists who observed the limbal cornea by slitlamp microscopy, first described this visible structural uniqueness of the limbal surface as "radial stripes", later as "limbal palisades of Vogt" (1). At the limbal zone, the Descemet's membrane is missing, too, and endothelial cells of the transitional zone (transitional cells) are larger and flatter than those in the central cornea.

In the human embryo, the corneal epithelium is formed by the 6th gestational week, from the surface ectoderm overlying the detaching lens (2). At this stage, it consists of a two-layered epithelium that is separated from the endothelium by an acellular space, the so called primary stroma (3). Studies of chick embryos proved that the corneal stroma and endothelium originates from neural crest derived mesenchymal cells (4), however, morphogenesis of the mesenchyma-derived tissues in the human cornea is not clear yet. In situ formation from mesenchymal tissue (5,6), as well as a three wave migration of neural crest cells giving rise to the iris, corneal endothelium and stromal keratocytes (2,7) have been proposed.

Adult, or, in newer terminology tissue-derived/resident stem cell populations are found in most adult tissues and are able to maintain and regenerate the given tissue for a lifetime. They are characterized by the following properties: (i) self-renewal, that is, during cell division, at least one of the daughter cells remains a stem cell; (ii) undifferentiated state, but high differentiation potential—implying the ability to differentiate into all cell types of their home tissue and possibly into other cell types as well, when appropriate (experimental) circumstances are provided; (iii) Slow cell cycle, that is, most of the time, stem cells are in a growth arrested state, however, they can enter cell cycle on demand (e.g., tissue injury), and give rise to a differentiating and highly proliferative progeny (progenitor cells); (iv) requirement for a stem cell niche—stem cells usually reside in a microenvironment that provides external factors necessary for maintaining stem cell properties and functions, often referred to as "stemness". For reviews see (8,9). Progenitor cells are similar to stem cells in most aspects except they do not renew themselves and thus their population becomes terminally differentiated after a limited, though sometimes enormous number of cell divisions. Although not a consensus yet, stem and progenitor cells are sometimes collectively termed precursors, indicating that both can be expanded under appropriate circumstances.

In the adult cornea, stem cells reside in the limbal area. The existence of epithelial stem cells in the limbus has been proposed in 1971 by Davanger and Evensen and the investigation of limbal epithelial stem cells (LESCs) greatly progressed since then. However, data suggesting the existence of stromal and endothelial stem cells in the cornea have only been published recently. In this review, we summarize the present state of knowledge on the stem cells of the adult cornea. Special emphasis is given to the latest progress in the molecular characterization of LESCs. Current information on putative stromal and endothelial stem cells is summarized as well.

Limbal Epithelial Stem Cells

Localization

Limbal localization of corneal epithelial stem cells is widely accepted today. Numerous experimental and clinical observations support this hypothesis.

In 1971, Davanger and Evensen observed pigmented epithelial migration lines moving from the limbal area toward the central cornea during the healing process and proposed that the limbal area could be the reservoir of the new epithelial cells (10). Later, cell movement was observed centripetally from the corneoscleral limbus during wound healing (11) and the measured median distance migrated by marked epithelial cells was about 17 microns per day (12).

The second evidence of limbal epithelial stem cells was provided by Cotsarelis who detected label-retaining cells in the basal layer of the limbal epithelium. Following pulse labeling of replicating DNA (with ^{3}H-thymidine or BrdU), these cells retain an easily detectable amount of label in their nucleus as opposed to frequently dividing cells in which the amount of label per cell decays quickly (13). Consecutive studies confirmed this finding, showing that the label retaining index (LI—percent of tritiated thymidin incorporating cells) is more than 20% in the limbal corneal region in contrast to the central cornea where LI is less than 8% (14,15).

Cell culture studies showed that cells from the central cornea generated mostly paraclones, i.e., terminated colonies which could not be passaged more than twice, whereas cells from the limbal area could proliferate for many generations (80–100 doublings) and formed large holoclone colonies. The peripherial cells formed meroclone colonies whose growth was stopped after a relatively small number of divisions (16–18). These cells are also referred to as transient amplifying cells (TACs).

In animal experiments, surgical removal of the limbus resulted in insufficient re-epithelization and conjunctival invasion of the corneal surface (19). Clinical studies have shown that limbal transplantation makes possible long-term restauration of the corneal surface in patients with limbal damage (20,21). Also, most epithelial tumors of the ocular surface originate from the limbal area (22–26).

Stem cells and their progeny exist millimeters apart in the human cornea (17). Limbal stem cells reside in the basal layer of the limbal epithelium and are interspersed with early transient amplifying cells (TACs). Cell differentiation occurs as cells migrate in two directions, toward the surface epithelium and toward the center of the cornea. Thus, TACs are found mostly in the peripheral cornea, and most cells are terminally differentiated in the central cornea, although the basal layer contains TACs, even centrally. The recognition of the distinct localization of stem cells and their progeny in the corneal epithelium, their easy accessibility on the ocular surface, as well as the transparency of the cornea allowing in vivo observation of the putative LESCs and their niche, has made the corneal epithelium an ideal system for the investigation of epithelial stem and transient amplifying cells (27). In addition, it allowed clinicians to develop corneal epithelial stem cell transplantation procedures for the treatment of so far non curable ocular surface diseases associated with limbal stem cell deficiency, even though a definitive LESC marker still remains elusive (28).

In the wounded cornea, such spatial separation of different cell types may not be apparent, at least when examined with the currently available methods. In rabbit models, Park et al. showed an increase in side population cell numbers in the limbal area on Day 1 after wounding, followed by an increase of colony forming efficiency of the central corneal cells on Day 5 (29). In a human cornea organotypic model, central corneal cells showed a faster proliferative response after wounding than limbal epithelial cells, and they could effectively regenerate central wounds in the absence of limbal epithelial cells (30). These results indicate that immediate wound healing response is mainly TAC function. Activation of LESCs takes more time, 24 h in rabbits and perhaps more in humans, and is followed by an additional increase of central corneal cell proliferation. It is not clear whether such an increase in proliferative potential is associated with a less differentiated phenotype of the central corneal cells.

Characteristics of Limbal Epithelial Stem Cells

Cell size and morphology. Resident stem cells in various adult tissues have an undifferentiated phenotype, and are characterized by small cell size and high nucleus to cytoplasm (N/C) ratio. Presumably, the nuclear size and DNA content is constant but the volume of cytoplasm is changed as new proteins start to appear in the differentiation process (31). Rowden detected a fourfold increase in cytoplasmic volume as cells passed from the basal to granular layers in the epidermis. During this transition, nuclear volume did not decrease significantly (32). In 1985, Barrandon showed that the size of keratinocytes determines their colony forming ability and keratinocytes larger than 20 μm lose their colony forming capacity (33). The same features seem to hold true for corneal epithelial stem cells.

In vivo confocal microscopic studies showed that limbal basal cells have smaller diameter (around 10 μm in all studies), larger N/C ratio, and have higher cell densities than central corneal or limbal suprabasal cell layers (34–37). Transmission electron microscopy showed small-roundish cells at the bottom of the palisade rete ridges with large nuclei containing heterochromatin-rich DNA and a barely detectable nucleolus, scarce cytoplasm with delicate melanin granules and a fine basement membrane through which cytoplasmic invaginations from the underlying stroma could be observed (38,39). In a recent study, cells with the smallest size (10–16 μm) expressed the highest amount of the putative stem cell markers ΔNp63 and ABCG2, both at mRNA and protein levels, included among themselves the most label-retaining (side population) cells, and had the highest clonogenic capacity in culture (40).

Slow cycling and self-renewal. Under normal conditions, stem cells are quiescent. They rarely undergo cell division, and

the resulting daughter cells, the transient amplifying cells (TACs) multiply to give rise to high numbers of differentiated cells, thus ensuring normal tissue homeostasis (8). This low mitotic activity of stem cells further protects their integrity by decreasing the possibility of DNA damage. During mitosis, stem cells renew themselves, e.g., at least one of the daughter cells preserves its "stemness". However, stem cells can exhibit an impressive proliferative capacity upon tissue injury or in culture (41–46). The slow cycling of limbal stem cells allowed their detection as label retaining cells (13).

Recently, the role of some molecules has been proposed in the self-renewal of LESCs. Barbaro et al. showed that CCAAT enhancer binding protein delta, C/EBPδ, colocalized with Bmi-1 and ΔNp63α in the limbal epithelial cells and identified quiescent LESCs that formed holoclones in culture. Forced expression of C/EBPδ reversibly inhibited the growth of limbal colonies and increased cell cycle length. C/EBPδ is suggested to exert its functions by activating the $p27^{kip1}$ and $p57^{kip2}$ genes and inhibiting the expression of $p^{16INK4A}$ and involucrin genes (47).

Plasticity. In accordance with the ectodermal origin of the corneal epithelium, differentiation of LESCs into various cell types of the epithelial and neuronal lineages has been demonstrated. Corneal cells could be differentiated into hair follicle cells when placed over hair forming embryonic dermis. Differentiation occurred in two steps: corneal cells first dedifferentiated to show limbal basal cell characteristics, then begun to express markers of epidermal differentiation and formed hair follicle-like structures (48–50). Subpopulations of limbal epithelial cells that acquired nestin positivity under the effect of mitogens in culture differentiated into functional neuronal cells (51,52). These cells could be further differentiated along the rod photoreceptor lineage in vitro and in vivo, suggesting that LESC can serve as a source for the treatment of retinal degenerative diseases such as age related macular degeneration or retinitis pigmentosa (53). The molecular basis of these transitions is not understood, although it was supposed that blocking the BMP signaling is important for neuronal differentiation of LESC (52) and the role of the Wnt/β-catenin pathway was suggested in differentiation into hair follicle cells (50,54).

In ex vivo cultured rabbit and human cornea limbal explants, limbal epithelial cells migrated into the stroma and showed changes suggesting that they underwent epithelial-mesenchymal transition (54,55). There is no evidence to support that such transitions could occur in vivo. However, the apparent plasticity of limbal epithelial cells in culture makes these cells a promising therapeutic tool for neurodegenerative and other diseases.

Molecular Markers of LESCs

Many molecules have been suggested to identify the basal cell layer of the limbal epithelium or clusters of cells within it and are thought to identify LESCs together with early TACs. On the other hand, differentiation markers that are not present in limbal basal epithelial cells can be used as negative markers for LESCs. Usually a combination of these markers is used for the identification of putative stem cells in the limbal epithelium. Positive LESC markers include cytokeratins Ck15, Ck14, Ck19, NGF receptor TrkA, vimentin, integrins α6, α9, β1, and β4. Negative markers are involucrin, connexins 43, and 50, along with cytokeratins Ck3 and Ck12 [reviewed in (56–62)]. A recent study has shown that integrin α9 positive cells are located adjacent to label retaining limbal basal cells, which in turn are negative for integrin α9. Therefore it was concluded that integrin α9 identifies cells in close proximity of the stem cells rather than LESCs themselves (63).

ΔNp63α. The p63 gene products are transcription factors whose role is essential in maintaining the cell populations that are necessary for epithelial development and morphogenesis. The p63 gene produces full length (TAp63) and N-terminally truncated (ΔNp63) transcripts, each of which have α, β, and γ isoforms (64). P63 was shown to identify basal cells with high proliferative potential in the skin (65). Later, high p63 content was showed in limbal epithelial cells with high N/C ratio and p63 was suggested as an LESC marker (18). It was also shown that antibodies detecting all isoforms of p63 identified differentiated cells as well (66). The isoform ΔNP63α was later shown to identify epithelial stem cells, whereas β and γ isoforms have been shown to promote epithelial cell differentiation (67,68). At present, ΔNp63α is considered as a reliable marker of both resting and activated limbal epithelial stem cells (47).

ABCG2. Similarly to hematopoietic (69), skin and muscle cells, limbal epithelial cells also yield a stem cell rich side population (SP) when sorted after incubation with the Hoechst33342 dye (70,71). SP phenotype has been attributed to the function of the BCRP/ABCG2 transporter protein (72–75). Cells from limbal explants expressing ABCG2 showed high clonogenic potential and expressed high levels of ΔNp63α, similarly to side population cells (76), therefore ABCG2 was suggested as a LESC marker. In histological sections, ABCG2 antibodies label small clusters of cells in the basal limbal epithelium (Fig. 2A), and $\sim$10% of the limbal epithelial cells are stained. On the other hand, only about 3% of the cells appear ABCG2 positive when measured by flow cytometry after isolation of single limbal epithelial cells by dispase II-trypsin digestion of corneas (76,77). Both numbers are higher than the proportion of LESCs, which is estimated to be less than 1% based on the fraction of the side population. These differences may be explained by cytoplasmic (nonfunctional) expression of ABCG2 in some limbal epithelial cells (77) and indicate that ABCG2 labeling with antibodies not only marks LESCs but possibly also some transient amplifying cells.

C/EBPδ and Bmi-1. Bmi-1 is a member of the polycomb gene family, and was shown to be essential for the self-renewal of haemopoietic and neural stem cells (78,79). In 2006, it was

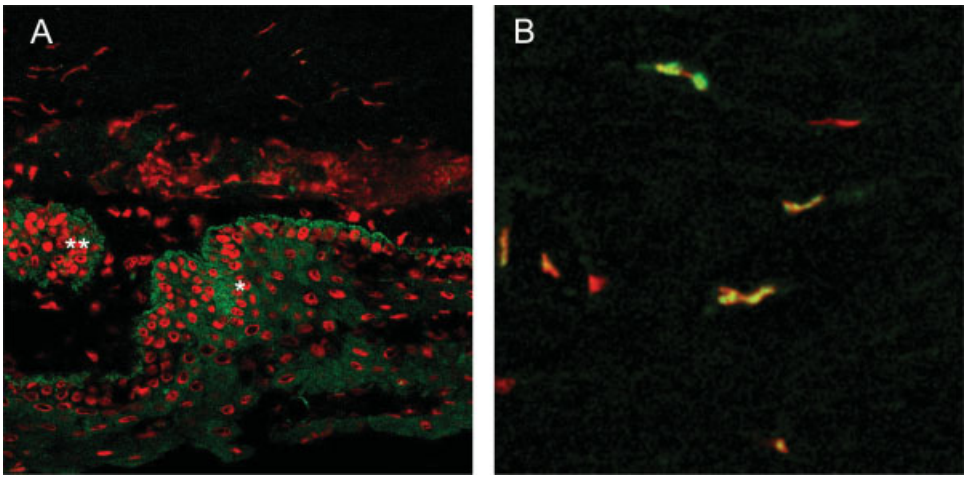

Figure 2. ABCG2 labeling of the palisades of Vogt in human corneal limbus. **A**: Basal epithelial cells of the palisades (*) and a putative limbal epithelial crypt (**). Field of view: 318 × 318 μm. **B**: Many stromal cells are also positive. Field of view: 94 × 94 μm. ABCG2: Green; nuclei (PI stain): red.

shown that Bmi-1 mRNA was expressed in the slow cycling side population cells of the limbal epithelium and was barely detectable in the central cornea, and it was suggested that Bmi-1 should be used as a LESC marker (80). In 2007, Barbaro et al. showed that C/EBPδ, together with Bmi-1 and ΔNp63α identifies resting limbal stem cells. C/EBPδ and Bmi-1 antibodies colocalized in histological sections and showed staining in ~10% of limbal basal epithelial cells. Upon activation (wounding), some of the cells switched off C/EBPδ and Bmi-1 expression but not ΔNp63α which continued to be expressed in proliferating basal epithelial cells (activated stem cells and TACs). Although Bmi-1 is a marker that can identify several types of adult stem cells (78,79), the action of C/EBPδ appears highly species and cell context specific and may not be exerted in other squamous epithelia apart from the limbal epithelium (47).

New potential stem cell markers identified in proteomic and mRNA profiling studies. The spatial separation of stem and transient amplifying cells in the limbal and central cornea allows an easy way to produce a stem cell enriched (limbal) and stem cell free (corneal) cell population. In recent years, several research groups, including ourselves, compared subsets of mRNAs and proteins expressed in the limbal and central cornea by the currently available high throughput nucleic acid profiling and proteomic techniques (81–87). Several of these molecules have been described as potential stem cell markers (Table 1). Immunohistology confirmed the presence of epiregulin, cytokeratins 14 and 15, p-cadherin, wnt-4, superoxide dismutase 2 (SOD2) and heatshock protein 70 (HSP70.1) in limbal basal epithelial cells. CK15, P-cadherin, wnt-4 and SOD2 identify small clusters of cells in the basal limbal epithelium that are probably closely related to LESCs. Cytokeratin 15 mRNA was found to be upregulated in the limbus by all but one of the cited studies, and also in hair follicle bulge stem cells (89,90). Although not confirmed by qRT-PCR or immunhistology, inhibitor of DNA binding molecule 4 (ID4), spondin-1, and catenin α2 mRNAs were found to be upregulated at least by two groups. ID4 was shown to promote G1/S phase transition in neuronal progenitors of the developing brain (91). Spondin-1 acts as a contact repellent molecule in neurogenesis (92). Catenin α2 regulates tight junction assembly (93). Interestingly, some molecules that have been shown to be upregulated in the limbus, were found in high amounts in the human amnion membrane by proteomic analysis. These are the basement membrane molecule collagen types VIα1 and α2; transglutaminase 2, a protein with various functions in extracellular matrix organization and cell-matrix interactions; as well as HSP70, superoxide dismutase, calizzarin, and integrin α6. These molecules may contribute to the cell growth promoting properties of human amniotic membrane, also used in corneal epithelial stem cell transplantation (94). Other molecules, such as tissue inhibitor of metalloproteinase 2 (TIMP2) and disabled 2 (Dab2) were shown in hair follicle bulge stem cells indicating a more general role of these molecules in epithelial stem cell regulation.

The Limbal Epithelial Stem Cell Niche

The particular microenvironment of stem cells (SCs), made up of extracellular matrix components, other resident cells and the products and signals they release, is known as a stem cell niche and is important for the modulation of SCs (95,96). A stem cell niche is also supposed to be a site where structural characteristics afford stem cell protection. Examining the niche in details requires the exact identification of SCs (97,98). Lack of specific LESC marker(s) renders the investigation of the limbal stem cell niche difficult.

Experiments indicate the role of the limbal stroma in stem cell niche formation. When limbal and corneal epithelial sheets were recombined with either limbal stroma or corneal stroma, the phenotype of the resulting epithelium unequivocally showed that limbal stroma modulated cell fate in the

Table 1. Putative molecular markers of corneal limbal epithelial cells from recent microarray studies

MOLECULE	SOURCE	REFERENCE
Confirmed presence in limbal basal cells by immunohistology		
Epiregulin	Frozen sections of mouse cornea, laser capture microdissection	(85)
Wnt-4	Human fetal cornea and primary cultures of adult human limbal epithelial cells	(87)
Cytokreatin 14	Human fetal cornea and primary cultures of adult human limbal epithelial cells	(87), Takács et al., unpublished
p-cadherin	Human fetal cornea and primary cultures of adult human limbal epithelial cells, human limbal epithelial scrapings	(87), Takács et al., unpublished
Cytokeratin 15	Human fetal cornea and primary cultures of adult human limbal epithelial cells, rat cornea	(81,84,87), Takács et al., unpublished
Superoxide dismutase 2	Human limbal epithelial scrapings	(81)
Molecules detected by at least two groups in mRNA expression arrays		
ID4	Human limbal epithelial scrapings	(88), Takács et al., unpublished
Spondin-1	Human limbal epithelial scrapings, frozen sections of mouse cornea	(85), Takács et al., unpublished
S100A8	Human limbal epithelial scrapings	(81), Takács et al., unpublished
Catenin-α2	Human fetal cornea and primary cultures of adult human limbal epithelial cells, frozen sections of mouse cornea	(85,87)
Molecules detected in limbal epithelial cells and also in human amniotic membrane		
Calizzarin	Rat limbal epithelial cells, intact and denuded human amniotic membrane	(82,84)
Collagen VI, chains α1, α2	Frozen sections of mouse cornea, intact and denuded human amniotic membrane	(82,83,85)
Transglutaminase 2	Rat cornea, denuded human amniotic membrane	(83,84)
Heat shock protein 70	Human limbal epithelial scrapings, intact and denuded human amniotic membrane	(81–83)
Superoxide dismutase	Human limbal epithelial scrapings, intact human amniotic membrane	(81,82)
Integrin α6	Human fetal cornea and primary cultures of adult human limbal epithelial cells, intact human amniotic membrane	(82,87)
Molecules detected in limbal epithelial cells and also in hair follicle bulge stem cells		
Tissue inhibitor of metalloproteinase 2	Frozen sections of mouse cornea, mouse hair follicle bulge cells	(85,89)
Disabled 2	Frozen sections of mouse cornea, mouse hair follicle bulge cells	(85,89)

direction of "stemness", while corneal stroma promoted differentiation and apoptosis (99). Niche structures are located in the limbal palisades of Vogt, which are radially oriented stromal ridges intersected with epithelial rete pegs, observable over the superior and inferior limbus, and missing temporally and nasally. The epithelium at the palisades is enriched in stem cells; targeted biopsies of limbal regions rich in palisades yield higher numbers of clonogenic LESCs in culture (37). In the region of limbal palisades, LESCs are in contact with a loose stroma without an intervening Bowmans' layer, thus, direct interaction of epithelial and stromal cells is possible. In the basal layers of the interpalisade rete ridges, small, round cells, exhibiting morphological features of primitive stem cells were shown (16,38,100,101). Protection of stem cells is thought to be fulfilled by three attributes of the palisades: they are situated in those parts of the cornea that are most protected by the eyelids; they contain melanocytes that safeguard stem cells from UV radiation by the transfer of melanin granules (35,38,102,103); in addition, protection from mechanical shear forces is provided at the bottom of the rete pegs. The palisade ridge regions contain blood vessels that can provide nutrients and other supportive factors for the SCs (1,39,58,101).

Recently, three anatomical structures were identified within the palisades of Vogt containing high numbers of putative stem cells, thus considered as putative stem cell niches.

One such structure, termed limbal epithelial crypt (LEC) was shown by serial sectioning of the limbal area. LECs appear as solid chords of epithelial cells, extended from the peripheral aspect of the undersurface of an interpalisade rete ridge and are continued either parallel with the ridge under the conjunctiva or circumferentiallly along the limbus, at right angles to the palisade. LECs contain high numbers of epithelial cells expressing the putative LESC markers ABCG2 (Fig. 2A, see the label **), p63, cytokeratins 14 and 19, vimentin and connexin 43, have a high nucleus to cytoplasm ratio and are connected to the underlying basement membrane via cytoplasmic projections (104,105).

Another study used in vivo confocal microscopy, as well as transmission and scanning electron microscopy to investigate human limbal structures. Two other putative niche structures, termed limbal crypts (LCs) and focal stromal projections (FSPs) were described in this latter study. LCs are circumscribed downward projections of the limbal epithelium that open to the corneal surface and are in close association with the limbal vasculature. FSPs are figerlike projections of stroma containing a central blood vessel that are surrounded by small, tightly packed epithelial cells. Highest numbers of p63α and ABCG2 positive epithelial cells, suspected as stem cells, were observed in the basal epithelial layers of LCs and FSPs (37).

Little is known about the molecular mechanisms controlling limbal niche functions. Cytokines and the interaction of cells with extracellular matrix components have been suggested to play an important role in niche regulation. According to their interaction with surrounding cells, cytokines in the limbus have been divided into three groups by Li and Tseng: type I cytokines are released by the epithelium and their receptors are found mainly in stromal cells (TGFβ, IL-1β, PDGFβ). Type two cytokines and their receptors are found both in stromal and epithelial cells (IGF1, TGFβ1, TGFβ2, bFGF). Type III cytokines are released by the stroma, whereas their receptors are found in the epithelium (KGF, HGF) (106). A recent study identified further type I (NGF, GDNF) type II (NT-3, NT-4) and type III (BDNF) cytokines in the human cornea (107). Interestingly, many of these factors (KGF, HGF, NGF, TGFβ1, TGFβ2, bFGF) have been identified in human amniotic membrane, which can support in vitro LESC growth in the absence of feeder cells.

Basement membrane components in the limbal area differ from those in the central cornea (108). The basement membrane in the limbal area shows increased immunoreactivity for laminins α1, α2, β1, and γ3, agrin, BM40/SPARC and tenascin-C, which colocalize with basal cells positive for ABCG2, p63, and CK19 (61). Some ECM components such as tenascin-R, chondroitin-sulphate, versican, and vitronectin were seen underneath the basement membrane of vimentin positive basal epithelial cells (61). These components are known to occur at places where epithelial-mesenchymal transitions may take place under physiological and pathological conditions (109,110). Interestingly, such epithelial-mesenchymal transition was observed during in vitro culturing of rabbit and human limbal corneal explants (54,55). It is not known at present whether such a transition might have any significance in vivo.

Isolation of LESC

Isolation by fluorescence activated cell sorting. Although a surface marker for limbal epithelial stem cells has not been defined yet, several methods have been used to isolate stem cells from the limbal epithelium by FACS. Isolation of a side population based on Hoechst33342 dye exclusion that comprised less than 1% of all the cells has been reported by several groups (29,70,76,77,80,111,112). Sorting the side population revealed species specific differences. In rats, a side population could be isolated also from the central cornea in addition to the limbal SP. However, central SP cells neither showed stem cell characteristics, nor were ABCG2 positive, indicating that several types of transporters may take part in Hoechst33342 dye efflux in the rat cornea (111). In addition, the presence of ABCG2 positive, slow cycling Langerhans cells was demonstrated in the limbal basal epithelium of rats, implying that cells sorted based on ABCG2 expression or Hoechst dye exclusion may yield a heterogeneous population of rat limbal stem cells (113).

In the human limbus, epithelial SP cells are slow cycling and express Bmi-1, nestin, and Notch-1 mRNA, indicating their quiescent stem cell phenotype (77,80,112).

Similar to keratinocytes, high integrin α6 and low CD71 expression identifies a subpopulation enriched in stem and progenitor cells among limbal epithelial cells. The integrin $\alpha6^{bright}$/$CD71^{low}$ cell population was rich in small cells, had a high clonogenic capacity, and expressed high levels of the stem cell markers ABCG2, Bmi-1, and ΔNp63 (114).

Most recently, a new cell surface marker, RHAMM/HMMR was described which was completely absent from cells of the basal epithelial layer of the limbus. Cell selection based on Hoechst exclusion and lack of cell surface RHAMM/HMMR expression resulted in increased colony forming efficiency compared to negative selection using RHAMM/HMMR alone or positive selection using Hoechst33342 on its own (115).

Isolation by centrifugation. In a recent work, mouse limbal epithelial cells were separated on a Percoll gradient. The densest fraction (less than 7% of original cells) contained small nonspontaneously proliferating cells, positive for p63 that acquired a high proliferative activity when cultured on a 3T3 feeder cell monolayer, indicating the presence of quiescent stem cells in this fraction (116).

Culturing of LESC

To maintain limbal epithelial cells in culture, a feeder layer of fibroblast cells (3T3 cells) is needed, similarly to other epithelial cells (117). For research use, culture on 3T3 cells in SHEM (supplemented hormonal epithelial medium, Table 2.) is the generally accepted method. 3T3 cells are either lethally irradiated or growth arrested with mitomycin C and then seeded on tissue culture dishes at 2×10^4 cells/cm^2 (117,118).

Table 2. Supplements of the SHEM medium for ex vivo expansion of limbal epithelial cells

Fetal bovine serum (%)	5–10
Patient's autologous serum (%)	**10**
EGF (ng/ml)	5–**10**
Insulin (ng/ml)	**2.5**–5
Transferrin (μg/ml)	5
Sodium selenite (ng/ml)	5
Hydrocortisone (μg/ml)	**0.1**–0.5
Cholera toxin subunit A (ng/ml)	30–**100**
DMSO (%)	0.5
Triiodothyronine (nmol/l)	2
Antibiotics	
Penicillin/streptomycin (IU/l) or	**10**
Gentamycin (μg/ml)	**50**
Antimycotic	
Amphotericin B (μg/ml)	**1.25**

The base medium is Dulbecco's minimal essential medium (DMEM) and Ham's F12 mixed 1:1. The components minimally necessary (119) for ex vivo expansion of limbal epithelial cells on denuded human amniotic membrane are set in bold.

The base medium is Dulbecco's minimal essential medium (DMEM) and Ham's F12 mixed 1:1. The components minimally necessary (119) for ex vivo expansion of limbal epithelial cells on denuded human amniotic membrane are listed in Table 2.

In the past 10 years, after the pioneering work of Pellegrini et al. (120), the transplantation of ex vivo expanded limbal epithelial cells is more and more widely used for the treatment of limbal stem cell deficiency. Methods and results of limbal epithelial stem cell transplantation have been reviewed elsewhere (28,121). In this section, we discuss some aspects of the cell culture methods used in clinical applications. When limbal epithelial cells are cultured for clinical use, it is intended that all animal derived products are excluded from culturing media, to avoid transmission of animal derived antigens or viral diseases. The use of human amniotic membrane instead of 3T3 cells, as well as replacement of FCS with the patients' autologous serum in the culture media was introduced by some groups (118,119,122). The human amniotic membrane (AM) may be intact (iAM, containing devitalized amniotic epithelium) or denuded (dAM, without epithelium) (123).

Several methods of culture are used in clinical applications. Explant cultures use little (2–3 mm^2) pieces of limbal tissue from biopsy that is placed on amniotic membrane (AM) fixed on a tissue culture insert or folded around a glass slide. The tissue culture dish may or may not contain devitalized 3T3 cells. Usually the first outgrowth of cells is used for transplantation, and the AM itself is used as a carrier in the transplantation process. In single cell cultures, epithelial cells are released form the limbal biopsy specimen by trypsin or combined dispase II-trypsin digestion (124). Subsequently, single cells are seeded either onto AM serving as a carrier, or onto growth arrested 3T3 cells. In the latter case, colonies of cells are passaged on 3T3 cells and, when confluent, dispase digested and placed on a carrier that is transplanted onto the ocular surface. Paraffin gauze, contact lenses (120), collagen shields (125), temperature sensitive biopolymers (126), fibrin gels (127,128), and anterior lens capsule (129) were used as carriers so far.

Although the basis of applying human amniotic membrane in transplantation procedures is its ability to support limbal epithelial cell growth in culture, this growth is arrested after three passages on intact AM, indicating that stem cells are not preserved on iAM (54,130). Probably the same applies for dAM as well, since limbal epithelial cells cultured on iAM showed a less differentiated phenotype (exhibiting less cytokeratin3, connexin43 and 50 expression by immunostaining and immunoblots) than those cultured on dAM, with an intermediate degree of differentiation of LESCs on dAM in the presence of 3T3 feeder cells (131). The evident, although limited growth supporting ability of human amniotic membrane may be owed to its growth factors and basement membrane components, many of which, including NGF, KGF, HGF, bFGF, TGFβ, and integrins β1 and β4 have been shown to be present in the limbal epithelium and stroma as well [reviewed in (123)]. Moreover, AM also has anti-inflammatory and anti-angiogenic effects (132). Recently, amniotic membrane was shown to induce overexpression of the IL-1 cytokine receptor antagonist (IL-1RA) in LESCs, and its anti-apoptotic effect was also demonstrated (133).

Using 3T3 cell layers may better preserve stem cells in single cell culture (118), however, in this case limbal epithelial cells are in contact with animal cells. Recently, a human amniotic epithelial cell line (134) and human embryonic fibroblasts (135) were successfully used as a feeder layer for LSCs, thus forecasting the possibility of generating corneal epithelial sheets for transplantation under better defined, standardized conditions without the use of animal material.

At present, the proportion of LESCs transplanted by any of the methods is not known, since epithelial sheets were not examined for the presence of stem cell markers, such as ΔNp63 or ABCG2 before transplantation. Similar clinical success rates with the use of the different culture techniques indicate that other processes, such as regeneration of the stem cell niche and repopulation by remaining stem cells, even perhaps by bone marrow derived cells, is possible. Regeneration of the host's own stem cell reservoir is supported by the fact that only the host's epithelial cells were found on the corneal surface 6–9 months after LESC allotransplantation (136).

Stromal Stem Cells

In 2005, isolation of murine and bovine corneal stromal stem cells by sphere forming assay was reported by two independent groups (137,138). In the same year, isolation of stromal stem cells from human cornea was also reported (139). In this latter study, some stromal cells have shown ABCG2 positivity (see also Fig. 2B). Based on this observation, the side population was selected from the cells released from the corneal stroma by digestion with collagenase and hyaluronidase. In culture, these side population cells showed clonal growth

and could be differentiated to express keratocyte, chondrogenic and neurogenic markers (139). The same group has concomitantly showed that while these undifferentiated corneal stromal stem cells predominantly express generic stem cell related genes (Bmi-1, Kit, Notch-1, Six2, Pax6, ABCG2, Spag10, p62/OSIL) in adherent cultures, when passaged in suspension in serum free medium with FGF2 and insulin, they form spheroid pellets, in which keratocyte-like cells secrete an ordered ECM and express mRNAs of known (keratocan, PTGDS, ALDH3A1) and potential (FLJ30046/SLAIN, CxAdR, PDK4, MTAC2D1, F13A1) keratocyte markers (140).

Multipotent, fibroblast-like cells were isolated from limbal stroma by other groups as well (141,142). In the earlier study, after enzymatic digestion of de-epithelized stroma of limbal explants, stage specific embryonal antigen 4 (SSEA-4) positive cells were sorted by MACS. The isolated multipotent fibroblast-like cells showed a unique marker profile ($CD34^-$, $CD45^-$, $CD123^-$, $Cd133^-$, $CD14^-$, $CD106^-$, $HLA\text{-}DR^-$/$CD31^+$, $SSEA\text{-}4^+$, $CD73^+$, $CD105^+$), different from that of bone marrow mesenchymal (143,144) or other adult stem cells but similar to that of embryonic stem cells ($Oct\text{-}4^+$, $Sox\text{-}2^+$, $Tra1\text{-}60^+$, $Tra1\text{-}81^+$) (141). This marker profile is quite similar to that of very small embryonic-like stem cells of the adult humans (46). The other group has propagated the fibroblast-like outgrowth from limbal explants which could be observed after removing the epithelial cells that preceded them in the outgrowth process. These fibroblast-like cells also formed spheroids in culture, were multipotent and exhibited a mesenchymal stem cell-like surface marker phenotype ($CD105^+$, $CD106^+$, $CD54^+$, $CD166^+$, $CD90^+$, $CD29^+$, $CD71^+$, $Pax6^+$/$SSEA\text{-}1^-$, $Tra1\text{-}81^-$, $Tra1\text{-}61^-$, $CD31^-$, $CD45^-$, $CD11a^-$, $CD11c^-$, $CD14^-$, $CD138^-$, $Flk1^-$, $Flt1^-$, $VE\text{-}cadherin^-$) (142).

The presence of bone-marrow derived cells in the cornea was shown when irradiated wild type mice were transplanted with bone marrow or hematopoietic stem cells of GFP expressing transgenic mice. Most of these cells differentiated into antigen presenting cells in the host's cornea and only a small percentage of BM derived cells represented other (unidentified) cell types (145,146). Bone marrow derived cells formed approximately half of the pericytes but none of the endothelial cells of the new vessels in a mouse model of experimental corneal neovascularization (147). Recently, bone marrow derived progenitor cells were shown to promote wound healing and re-epithelization in alkali injured rabbit corneas (148,149).

Yoshida et al. (138) isolated a subset of cells termed neural crest derived corneal precursors (COPs) from stromal cells of adult mice. These cells showed side population characteristics, were multipotent, clonogenic (sphere forming), and expressed various adult stem cell markers (nestin, Notch-1, Musashi-1, ABCG2). Experiments with transgenic mice proved that limbal bone marrow derived cells and COPs are two distinct cell populations and that COPs have a neural crest origin, which was also confirmed by the expression of the embryonic neural crest markers Twist, Snail, Slug and Sox-9. COPs expressed surface markers Sca-1 and CD34 and were negative for CD45 and c-kit.

Altogether, these results indicate that bone marrow derived cells mainly act as enhancers of wound healing and neovascularization, and take part in the immunological defense of the cornea. On the other hand, corneal stromal stem cells and COPs may serve as stem cells in the maintenance of the mesenchyma-derived parts of the cornea. As both cell types are located mainly in the peripheral cornea, interactions between them are possible. Understanding these interactions, as well as elucidating the behavior of these cell types under physiological and pathological conditions will greatly increase our knowledge on corneal wound healing and regeneration.

Endothelial Stem Cells

Although human endothelial cells can be grown in culture, it is believed that these cells cannot undergo cell division beyond the age of 20 in vivo (150). Nonetheless, starting from 2005, some studies indicating the presence of pluripotent precursors/putative stem cells in the human corneal endothelium have been published. In 2005, Whikehart observed that peripheral corneal endothelial cells incorporate BrdU indicating mitotic activity, moreover, the number of BrdU incorporating cells increased upon wounding (151). In a sphere forming assay, Yokoo et al. isolated endothelial cell colonies from human corneal endothelial cells that expressed neuronal and mesenchymal markers. These cells had a limited self-renewing capacity as indicated by failure to form spheres by the third passage, therefore are considered as progenitors. Differentiated adherent progeny of the sphere colonies cultured on fetal bovine endothelium ECM showed corneal endothelial cell-like morphology and pump functions (152). Amano et al. isolated similar sphere colonies from the human and rabbit endothelium and showed that the sphere forming capacity of the peripheral endothelium was significantly higher compared to the central endothelium in rabbits (153). In 2007, McGowan et al. showed that cells from the trabecular meshwork and peripheral endothelial cells (transition zone) expressed nestin and telomerase and further stem cell and differentiation markers (oct-3/4, wnt-1, Pax-6, Sox-2) were seen in these structures after wounding (154). These experiments strongly support the existence of endothelial stem cells, residing perhaps in the trabecular meshwork and the periphery of the corneal endothelium.

The factors that inhibit proliferation of these cells in vivo are not fully understood at present, although the role of contact inhibition and TBFβ2 has been proposed (155). Transport functions of the endothelium are essential in maintaining corneal clarity. The in vivo observed postmitotic properties of this tissue make it highly vulnerable during surgical manipulations, and corneal endothelial cell loss due to cataract surgery or degenerative diseases is a leading cause of corneal transplantations. Hence, the pathways regulating endothelial quiescence versus proliferation have great potential as eventual therapeutic targets.

Based on the sphere forming properties, common neural crest origin, and the presence of some markers common with COPs and corneal stromal stem cells (nestin, oct-3/4, Pax-6, Sox-2), it can be hypothesized that corneal endothelial and stromal stem cells/COPs may form a common reservoir in the

Table 3. Major stem cell types found in the adult cornea

STEM CELL (SC) TYPE	DEVELOPMENTAL ORIGIN	LOCALIZATION	ISOLATION/CULTURE	DIFFERENTIATION POTENTIAL	MARKERS
Limbal epithelial stem cells (LESCs)	Ectoderm	Basal layer of limbal epithelium, palisades of Vogt	Explant cultures of corneal limbus or single limbal epithelial cells on feeder 3T3 cells	Hair follicle cells, neuronal cells, possible mesenchymal transition in limbal explants	No definitive marker ABCG2 and ΔNp63α are accepted as putative markers, Bmi-1 and C/EBPδ mark quiescent stem cells
Stromal stem cells	Neural crest derived mesenchyme	Central and peripheral corneal stroma, mostly the peripheral part	Side population of single stromal cells	Chondrocytes, neuronal cells	No definitive marker differential expression of Bmi-1, Kit, Notch-1, Six2, Pax6, ABCG2, Spag10, Osil mRNAs
Stromal fibroblast-like cells	Not known	Stroma of limbal explants	SSEA-4$^+$ cells isolated by MACS	Neuronal cells, corneal epithelial cells, chondrocytes, myocytes, osteoblasts, adipocytes, pancreatic cells, hepatocytes	FACS: CD31$^+$, SSEA-4$^+$, CD73$^+$, CD105$^+$, CD34$^-$, CD45$^-$, CD123$^-$, Cd133$^-$, CD14$^-$, CD106$^-$, HLA-DR$^-$ immunofluorescence: Oct-4$^+$, Sox-2$^+$, Tra1-60$^+$, Tra1-81$^+$
Stromal mesenchymal cells	Not known	De-epithelized limbal explants	Spontaneous spindle cell outgrowth of limbal explants	Adipocytes, Osteocytes	FACS: CD105$^+$, CD106$^+$, CD54$^+$, CD166$^+$, CD90$^+$, CD29$^+$, CD71$^+$, Pax6$^+$ SSEA-1$^-$ Tra1-81$^-$, Tra1-61$^-$, CD31$^-$, CD45$^-$, CD11a$^-$, CD11c$^-$, CD14$^-$, CD138$^-$, Flk1$^-$ Flt1$^-$, VE-cadherin$^-$
Neural crest derived corneal stem cells (COPs)	Neural crest	Mouse corneal stroma	Sphere forming assay	Keratocytes, chondrocytes, adipocytes, neural cells	FACS: Sca-1$^+$, CD34$^+$, CD45$^-$, c-kit$^-$ Immunofluorescence: ABCG2$^+$, Musashi-1$^+$, nestin$^+$, wnt-1$^+$
Putative endothelial stem cells	Neural crest	Peripheral endothelium	Sphere forming assay, IHC detection in corneal buttons	Corneal endothelial cells, express mesenchymal and neuronal proteins	Immunofluorescence: oct-3/4$^+$, wnt-1$^+$, Pax-6$^+$, Sox-2$^+$, nestin$^+$, telomerase$^+$

cornea, and transition from one lineage to the other might be relatively easy. However, there is no experimental evidence for this as yet. A summary of the major stem cell types found in the adult cornea is provided in Table 3.

Perspectives

Corneal stem cell research has seen great advances in the past few years. The use of cultured limbal epithelial stem cells in the treatment of ocular surface diseases has become a widely accepted method. To improve the results of this intervention, as well as to properly exploit the recent advances of corneal stromal and endothelial stem cell research, many questions have to be answered. It is not known at present, what percentage of the transplanted cells are stem cells during the transplantation of ex vivo expanded limbal epithelial cells. The fact that host derived epithelial cells cover the corneal surface of grafted patients after 9 months raises the possibility that not the stem cells themselves, but reestablishment of the host's stem cell niche or reactivation of his/her own damaged stem cells is somehow involved in the healing process (136). To understand these phenomena, a better knowledge of the corneal epithelial stem cell niche functions, as well as elucidation of the role of bone marrow derived cells in supporting corneal wound healing is necessary.

Development of new therapeutic strategies that bring into play the regenerative potential of cornea specific stromal precursor cells may provide therapeutic tools that make possible the regeneration of corneal ulcers and injuries with transparent, avascular scars. The use of corneal stromal stem cells in tissue ingeneering as components of artificial corneas is an intriguing possibility, too (156). Endothelial replacement by posterior deep lamellar keratoplasty from heterologous tissue is an ongoing surgical procedure. The use of the patient's own endothelial stem cell reservoir to produce transplantable endothelial sheets may reduce the risk of rejection and decrease difficulties related to immune suppression. The apparent flexibility of the different types of corneal stem cells may make them a useful therapeutic tool in the treatment of degenerative diseases of neural derived tissues of the eye, as well as of other organs such as the brain, and perhaps even of the heart or the pancreatic islets (141). On the other hand, stem cells from other organs may serve as autologous sources of replacement in ocular surface diseases, as has been shown in the case of bone marrow mesenchymal cells, adult epidermal or oral mucosal stem cells (126,132,157).

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