Review

The Limbal Epithelial Progenitors in the Limbal Niche Environment

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Abstract

Limbal epithelial progenitors are stem cells located in limbal palisades of Vogt. In this review, we present the audience with recent evidence that limbal epithelial progenitors may be a powerful stem cell resource for the cure of human corneal stem cell deficiency. Further understanding of their mechanism may shed lights to the future successful application of stem cell therapy not only to the eye tissue, but also to the other tissues in the human body.

Key words: Limbal epithelial progenitors, stem cell

Introduction

The human eye, a window to the world, is our important photoreceptive organ. A healthy surface of the eye is critical for proper vision. The anterior surface, usually called ocular surface, is defined by the cornea that is surrounding by conjunctiva. And the important transition zone between them is limbus [1]. The cornea, which forms the central region of the ocular surface, provides more than two-thirds of the eye’s refractive power. And it also serves a protective role by providing the defense against desiccation, infection and injury [2]. During the eye development, human cornea is one of the last structures being formed. The human cornea is a lamellar-structured tissue comprised by five layers. The anterior cornea is composed of non-keratinized squamous epithelium. The substantia propria containing collagenous and avascular stroma is sparsely populated with keratocytes (fibroblasts). The inner part is a monolayer tissue termed endothelium. Interestingly, corneal stromal keratocytes and endothelial cells are all derived from the neural crest. Each part is separated by a membrane, anteriorly by Bowman’s layer and posteriorly by Descemet’s membrane [3, 4]. The corneal epithelium is further divided into three layers: basal, wing, and squamas. Basal cells secrete matrix molecules, which is a composition of the basement membrane (BM). Squames can protect against external environment by forming lateral tight junctions, and wing cells play a role in wound healing [5]. The conjunctiva, which is divided into three zones (bulbar, fornical and palpebral), is a loose and vascularized tissue between sclera and the epidermis of the eyelids [6]. The conjunctiva’s most important functions are secretory, facilitated by goblet cells and immune related, carried out by its resident Langerhans cells [7].

Limbal Epithelial Stem Cells

Stem cells are undifferentiated cells that can be able to provide an unlimited supply of proliferating cells. A large body of research indicated that there is a stem cell pool reside in the limbal basal region named
limbal epithelial stem cells (LESC). LESC share several features with other somatic stem cells, including small cell size [8], high nuclear to cytoplasmic ratio [9], and lack expression of differentiation markers [10, 11]. The key characteristics of stem cells are high capacity for self-renewal and poor differentiation. They have long cell cycle time, long life span, error-free proliferation, and the ability to divide in an asymmetric way. Asymmetric division allows one of the daughter cells to maintain stemness and replenish the stem cell pool, while the other daughter cell becomes a “transient amplifying cell” (TAC) that follows the path of differentiation. Transient amplifying cells which have a limited proliferative potential can divide more frequently than stem cells [9]. After differentiation, these cells become “post-mitotic cells” and finally, “terminally differentiated cells”, both of which are incapable of division [12].

Accumulative evidence support limbus is the location of LESC. The first experimental evidence for the location of LESC was the movement of pigment from the limbus towards an epithelial defect in rabbit wound healing model [13]. Later, Davanger [1] observed a similar migration and proposed that the Palisades of Vogt (PV) situated in the limbus provided the source of LESC [14]. This movement has been described as centripetal migration. And this migration results in corneal neovascularization, impaired corneal function and conjunctival ingrowth [14]. Cotsarelis et al. [15] revealed that [3H] thymidine labeling could retained in limbal basal epithelial cells (LBEC) for long periods of time, indicating a long cell cycle. LBEC was also found to have higher mitotic activity than central corneal epithelial cells [16, 17]. This population which are small and round appear to be more primitive [8]. Another evidence is that complete [14, 18] or partial [19, 20] removal of limbal epithelium can lead to abnormal corneal wound healing, and the transplantation of LESC can improve epithelial healing.

The limbal basal region is rich in stem cell markers and lack of differentiation markers. Cytokeratin 19 (CK19) is a marker expressed in both limbal basal cells and conjunctival epithelial cells [6]. ΔNp63α, well known as a progenitor cell marker, was identified in the LESC using western blot [21]. ΔNp63α and ABCG2 expressed in the floating spheres obtained from human central corneal cells [22]. ABCG2 was also found to be expressed increasingly from central cornea to peripheral cornea and finally the limbus [22, 23]. Cytokeratin15 (CK15) is a stem cell marker which is specifically expressed in limbal basal epithelial cells [24, 25]. Other examples are differentiation markers cytokeratin 3(CK3), cytokeratin 12 (CK12) and connexin 43. Stroma in central cornea promoted expression of CK3 while stroma in limbus suppressed it. Limbal basal cells and the adjacent conjunctiva were lack of CK3 [10]. The similar pattern was found in CK12, the corneal specific protein [26]. Connexin 43 only expressed when corneal epithelium was cultured with corneal stroma [27]. However, various scientists used different markers to isolate and characterize native limbal epithelial progenitor cells (LEPC) (Table 1).

Table 1. Markers used to isolate and characterize natively exist LEPC.

| Author and year | Tissue | Markers to isolate | Markers to characterize |
|-----------------|-------|-------------------|------------------------|
| Ingram, 2005 [28] | Human umbilical vein or aortic endothelium | ND | ND |
| Werner, 2003 [29] | Mouse spleen | PKH-26 | ND |
| Bearzi, 2009 [30] | Human myocardium | Flik-1 | ND |

ND: Not Defined.

Apart from those natively existing LEPC in the perivascular niche, LEPC could differentiate from ESC in vitro, with the markers used various from study to study (Table 2), implying a highly heterogeneity of such multipotent progenitor cells. LEPC can be differentiated from LESC spontaneously when cultured in vitro [31], while the presentation of BMP4 could promote such differentiation dramatically [32, 33] [31]. LEPC could be further differentiated into LECs (Table 2). It remains unclear whether limbal stromal niche cells, which is believed to be derived from LNCs expressing LESC markers, can differentiate into LEPC and pericytes, and whether such differentiation requires BMP4 signaling.

The induction from LEPC to LEC in vitro, focus on medium and surface, have been summarized in Table 3.

**Limbal Stem Cell Niche**

Stem cell (SC) niche is defined in a highly specialize microenvironment consist of cellular components of extracellular matrix (ECM) and secreted growth factors. Collagenase can, but disperse cannot, isolate the entire limbal basal epithelial progenitors and subjacent mesenchymal cells from the limbal stroma [38-40]. In addition, collagenase in MESCM is the best known method to isolate the LNCs because collagenase in MESCM maintains the expression of the SC markers in fresh isolated LNCs [39]. Furthermore, the collagenase isolated limbal SCs as well as surrounding stromal cells, which are
identified as niche cells that support SCs [38-44]. These isolated vimentin+ LNCs express embryonic and other SC markers and have a differentiation potential into vascular endothelial progenitors [41] and mesenchymal stem cells which can differentiate into osteoblasts, chondrocytes, and adipocytes [41]. Interestingly, these cells also possess the pericyte phenotype to stabilize the vascular tube-like network formed by HUVEC in 3D Matrigel [41]. The progenitor status of LNCs [39] and their close contact [38, 40] with LEPC is critical to prevent corneal differentiation and to retain the limbal epithelial progenitors. Cell aggregation may lead to mesenchymal condensation as the first step of chondrogenesis and subsequent osteogenesis [45-47]. Aggregation of human mesenchymal stem cells (MSCs) into 3D spheroids enhances the effect of chondrogenesis and subsequent osteogenesis [45-47].

The limbal SC niche (LSCN) has both anatomic and functional dimensions. It is important and necessary to know where LSCN is before functional dimension is addressed. Anatomically, the LSCN is located at a wave-like structure called “Palisades of Vogt”. It has an undulated appearance with invaginations and projections into the deeper layers of the corneoscleral rim around cornea and also, with basal lamina structures. These structures are called limbal crypts [51], which provide a specific environment for limbal stem cells. This structure is highly pigmented due to the presence of melanocytes [1, 52, 53]. Similar to the function of human skin bulge area, melanocytes here may produce melanin pigments and transport it to epithelial cells, which can minimize ultraviolet irradiation damage [54]. Moreover, Palisades of Vogt is surrounded by a vascular network [54] which enables the infiltration of suppressor T-lymphocytes [55] and antigen-presenting Langerhan’s cells [56].

The highly vascularized structure provides the SC with nutrient and oxygen [57]. Unlike that of the cornea, the percentage of limbal basal cell membranes with hemidesmosomes was significantly less [58]. And the basement membrane of the limbus is undulating with papillae of stroma extending upward [58] and fenestrated [51, 59]. These features suggest that LESC might interact with underlying limbal stroma cells closely.

### Table 2. Induction from ESC to EPC and mature ECs (conditions and markers)

| Author and year | Origin | From LESC to LEPC | Inducer | Markers | From LEPC to mature LECs | Inducer | Mature ECs identifying assay |
|-----------------|--------|-------------------|---------|---------|--------------------------|---------|----------------------------|
| Park 2004 [34] | Human  | EGM-2             | PDGF    | Flk-1, CD31 | hybridoma medium         | BMP4 VEGF | CD31, CD34 and Flk-1       |
| Ferreira 2007 [31] | Human  | EGM-2             | PDGF    | Flk-1, CD31, CD133 | EGM-2 | VEGF | CD31, CD34 and Flk-1 |
| Lee 2008 [35]   | Murine | N2B27 medium      | PDGF    | Flk-1, CD31, CD133 | Methyl-cellulose medium | VEGF | CD34 Flk1 |
| Purpura 2008 [36] | Human  | N2B27 medium      | PDGF    | Flk-1, CD31, CD133, CD144 | EGM-2 cytokine N2B27 | VEGF | CD31, CD133 |
| Goldman 2009 [33] | Human  | EGM-2             | PDGF    | Flk-1, CD31, CD133, CD144 | BMP4 | hFGF2, VEGF-A165 BMP4 | CD31, CD133 |
| Noghero 2011 [37] | Murine | EGM-2             | PDGF    | Flk-1, CD31, CD133, CD144 | BMP4 | Flk-1 | CD31, CD133 |
| Park 2010 [32]   | Human  | EGM-2             | PDGF    | Flk-1, CD31, CD133, CD144 | VEGF bFGF | CD31, CD144 |

### Table 3. Induction from LEPC to LEC in vitro, focus on medium and surface

| Author and year | Origin | Induction of LEPC to LEC | Inducer | Medium Base | GFs | Surface | Mature EC Assay |
|-----------------|--------|--------------------------|---------|-------------|-----|---------|----------------|
| Goldman 2009 [33] | Human  | EGM-2                     | PDGF    | EGM-2 medium | VEGF 50ng/ml | 24well plate with coated Matrigel | CD31, CD144, CD34 |
| Park 2010 [32]   | Human  | EGM-2                     | PDGF    | EGM-2 medium | VEGF bFGF | Typical morphologies, express CD31, CD144, vWF, form vascular like structure on Matrigel, and took up acegylated-LDL | CD31, CD144, CD34 |

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Little is known about the characteristics of the primary precursor cells in vivo, since it has not yet been possible to isolate the most primitive mesenchymal cell from bulk cultures. One of the hurdles has been the inability to prospectively isolate MSCs because of their low frequency and the lack of specific markers. Recently, some groups have reported the identification and prospective isolation of the most primitive mesenchymal progenitors, both in murine and human adult BM, based on the expression of specific markers like SSEA-1, SSEA-3, SSEA-4, STRO-1, the low affinity nerve growth factor receptor (CD271), mesenchymal stem cell antigen-1 (MSCA-1), CD56 and PDGFR-β. (Table 4) Despite the identification of these new MSC markers, none of the markers are the true characteristic mesenchymal progenitors. Indeed, MSCs may be composed by different cell subsets which might be responsible for specific functions and characterized by different cell surface markers. Therefore, further research in this field is warranted in order to identify an MSC-specific marker; this will hopefully allow to dissect the developmental hierarchy of MSCs and will facilitate the generation of homogenous cellular products [60]. However, CD271^bright/PDGFR^- bone marrow derived cells has been proved to have the ability to give rise to genuine MSC in culture [62, 63].

A population of limbal NCs from collagenase-digested clusters and cultured on plastics coated with Matrigel in modified ESCM (ESCM plus 4ng/ml bFGF and 10ng/ml LIF), termed MESCM, was successfully used for expansion. Such expanded limbal NCs at P4 could reversible express EPC markers, when reseeded on 3D Matrigel. Specifically, they restored expression of all ESC markers, but further elevated expression of CD34, which is an important marker for angiogenesis progenitors [32, 67, 69]. Dravida et al [70] isolated limbal fibroblast-like cells (LFLC) from the human limbal explants using SSEA4 magic beads and noted that LFLC does not express CD34 while 90% of the LFLC express CD31, suggesting that such expanded cells on coated Matrigel might turn into EPC. Dravida used SSEA4 magnetic beads to select LFLC, and cultured them on 1% Matrigel coated plate. In contrast, we expanded the limbal NCs directly from collagenase digested clusters using 5% Matrigel coated plate. As mentioned in introduction, both LEPC and pericytes could be induced from ESC if given the appropriate condition, thus we speculated that 3D Matrigel could help induce limbal NCs expanded from collagenase digested clusters into angiogenesis progenitors, i.e. LEPC and pericytes.

**Conclusion**

Limbal epithelial progenitors are corneal epithelial stem cells, a powerful stem cell resources for cure of human corneal stem cell deficiency. Further studies of their mechanism are required for the future successful application of stem cell therapy to human eye diseases. If successful, such research may impact on the entire field of stem cell research and their clinical applications.

**Competing Interests**

The authors have declared that no competing interest exists.

**References**

1. Davanger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. Nature. 1971; 229: 560-1.
2. Kolozsvari I, Nogradi A, Hopp B, Bor Z. UV absorbance of the human cornea in the 240- to 400-nm range. Invest Ophthalmol Vis Sci. 2002; 43: 2161-8.
3. Nakatsu MN, Ding Z, Ng MY, Truong TT, Yu F, Deng SX. Wnt/beta-catenin signaling regulates proliferation of human cornea epithelial stem/progenitor cells. Invest Ophthalmol Vis Sci. 2011; 52: 4734-41.
4. Davis J, Duncan MK, Robison WG, Jr., Piatigorsky J. Requirement for Pax6 in corneal morphogenesis: a role in adhesion. J Cell Sci. 2003; 116: 2157-67.
5. Stepp MA, Zieske JD. The corneal epithelial stem cell niche. Ocul Surf. 2005; 3: 15-26.
6. Ang LP, Tan DT. Ocular surface stem cells and disease: current concepts and clinical applications. Ann Acad Med Singapore. 2004; 33: 576-80.
7. Inatomi T, Spurr-Michaud S, Tisdale AS, Zhan Q, Feldman ST, Gibson IK. Expression of secretory mucin genes by human conjunctival epithelia. Invest Ophthalmol Vis Sci. 1996; 37: 1684-92.
8. Romano AC, Espana EM, Yoo SH, Budak MT, Wolosin JM, Tseng SC. Different cell sizes in human limbal and central corneal basal epithelia measured by confocal microscopy and flow cytometry. Invest Ophthalmol Vis Sci. 2003; 44: 5125-9.

**Table 4. CD34+ or PDGFR-β+ are identified as typical MSC progenitor markers**

| Author and year | Citations |
|-----------------|-----------|
| Corselli 2012   | These novel MSC ancestors, which have been typified as CD34+CD146- cells, can differentiate in culture into CD34-CD146- pericytes. |
| Katare 2011     | CD34+ cells, located around the vasa vasorum in the adventitia of arteries and veins, also express typical pericyte markers (NG2, PDGFRβ, and RGS5) together with mesenchymal (CD44, CD90, CD73, CD29) and stemness antigens (Oct-4and Sox-2). This adventitial subset contains progenitor cells that may contribute to angiogenesis. |
| Campagnolo 2010 | Total vessel wall cell isolates contain CD34+/CD31- cells which upon culture express pericyte/mesenchymal markers. Integrate into vascular networks in vitro and in vivo |
| Traktuev 2008   | A population of multipotent CD34+ positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. |
| Schwab 2007     | CD146+/PDGFR-β+ cells from human endometrium underwent differentiation into adipogenic, osteogenic, myogenic and chondrogenic lineages. |
9. Purpura KA, Morin J, Zandstra PW. Analysis of the temporal and concentration-dependent effects of BMP-4, VEGF, and TPO on development of embryonic stem cell-derived mesoderm and blood progenitors in a defined, serum-free media. Exp Hematol. 2008; 36: 1186-98.

10. Nogueira A, Arese M, Bussolino F, Guandalini A. Mature endothelium and neurons are simultaneously derived from embryonic stem cells by 2D in vitro culture system. J Cell Mol Med. 2011; 15: 2200-15.

11. Chen SY, Hayashida Y, Chen MY, Xie HT, Tseng SC. A new isolation method of human limbal progenitor cells by maintaining close association with their niche cells. Tissue Eng Part C Methods. 2011; 17: 537-48.

12. Xie HT, Chen SY, Li GG, Tseng SC. Isolation and expansion of human limbal stromal niche cells. Invest Ophthalmol Vis Sci. 2012; 53: 279-86.

13. Xie HT, Chen SY, Li GG, Tseng SC. Limbal epithelial stem/progenitor cells attract stromal niche cells by SF-D1/CXCR4 signaling to prevent differentiation. Stem Cells. 2011; 29: 1784-5.

14. Li GG, Zhu YT, Xie HT, Chen SY, Tseng SC. Mesenchymal stem cells derived from human limbal niche cells. Invest Ophthalmol Vis Sci. 2012; 53: 5866-97.

15. Chen SY, Mahable M, Tseng SC. Optimization of Ex Vivo Expansion of Limbal Epithelial Progenitors by Maintaining Native Niche Cells on Demed Amniotic Membrane. Transl Vis Sci Technol. 2013; 2: 1.

16. Han B, Chen SY, Zhu YT, Tseng SC. Integration of BMP/Wnt signaling to control clonal growth of limbal epithelial progenitor cells by niche cells. Stem Cell Res. 2014; 12: 562-73.

17. Chen SY, Han B, Zhu YT, Mahable M, Huang J, Beebe DC, et al. HC-6A/IPX3 Purified From Amniotic Membrane Promotes BMP Signaling in Limbal Niche Cells to Maintain Quiescence of Limbal Epithelial Progenitor/Stem Cells. Stem Cells. 2015; 33: 3341-55.

18. Erbacher A, Filvaroff EH, Gitelman SE, Derynick R. Toward a molecular understanding of skeletal development. Cell. 1995; 80: 371-8.

19. MacLean HE, Kim JJ, Glimcher MJ, Wang J, Kronenberg HM, Glimcher LH. Absence of transcription factor c-maf causes abnormal terminal differentiation of hypertrophic chondrocytes during endochondral bone development. Dev Biol. 2003; 262: 51-63.

20. Kronenberg HM. Developmental regulation of the growth plate. Nature. 2000; 407: 422-32.

21. Bartosh TJ, Ylostalo JH, Mohammadipoor A, Bazhanov N, Coble K, Claypool K, et al. Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties. Proc Natl Acad Sci U S A. 2013; 110: 17242-9.

22. Xie T, Li L. Stem cells and their niche: an inseparable relationship. Development. 2007; 134: 2001-6.

23. Fuchs E, Tumbar T, Gazit Y. Socializing with the neighbors: stem cells and their niche. Cell. 2004; 116: 519-31.

24. Dua HS, Sharmunghanathan VA, Powell-Richards AO, Tighe PJ, Joseph A. Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. Br J Ophthalmol. 2005; 89: 529-32.

25. Dorsaz MA, Armer HE, Li A, Liu B, Li Y, Jaffe A, et al. Localization of epithelial cells capable of holoclonal formation in vitro and direct interaction with stromal cells in the native human limbal crypt. PLoS One. 2014; 9: e94283.

26. Gipsk OP. The epithelial basement membrane zone of the limbus. Eye (Lond). 1989; 3 (Pt 2): 132-40.

27. Goldberg MF, Bron AJ. Limbal palisades of Vogt. Trans Am Ophthalmol Soc. 1982; 80: 155-71.

28. Vannappan L, Geboes K, Missotten L, Maudgal PC, Desmet V. Lymphocytes and Langerhans cells in the normal human cornea. Invest Ophthalmol Vis Sci. 1985; 26: 220-5.

29. Baum JL. Melanocytes and Langerhans cell population of the cornea and limbus in the albino animal. Am J Ophthalmol. 1970; 69: 669-78.

30. Dorsaz MA, Armer HE, Li A, Liu B, Li Y, Jaffe A, et al. Localization of epithelial cells capable of holoclonal formation in vitro and direct interaction with stromal cells in the native human limbal crypt. PLoS One. 2014; 9: e94283.

31. Gipsk OP. The epithelial basement membrane zone of the limbus. Eye (Lond). 1989; 3 (Pt 2): 132-40.

32. Sharmunghanathan VA, Foster T, Kulkarni BB, Hopkinson A, Gray T, Powe DG, et al. Morphological characteristics of the limbal epithelial crypt. Br J Ophthalmol. 2007; 91: 514-9.

33. Bernardo ME, Corneta AM, Pagliara D, Roja MI, Fuentes R, Llinas R, et al. VWFA1/CD146 expression in corneal epithelial progenitor cells. J Cell Biol. 2008; 181: 303-13.

34. Chen CW, Monteleone E, Crisan M, Corsetti M, Huard J, Lazzari E, et al. Perivascular lining of mesenchymal stem cells in multiple human organs. Cell Stem Cell. 2009; 5: 303-13.

35. Chen CW, Monteleone E, Crisan M, Corsetti M, Huard J, Lazzari E, et al. Perivascular lining of mesenchymal stem cells in multiple human organs. Cell Stem Cell. 2009; 5: 303-13.
66. Campagnolo P, Cesselli D, Al Haj Zen A, Beltrami AP, Krankel N, Katare R, et al. Human adult vena saphena contains perivascular progenitor cells endowed with clonogenic and proangiogenic potential. Circulation. 2010; 121: 1735-45.

67. Traktuev DO, Merfeld-Clauss S, Li J, Kolonin M, Arap W, Pasqualini R, et al. A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. Circ Res. 2008; 102: 77-85.

68. Schwab KE, Gargett CE. Co-expression of two perivascular cell markers isolates mesenchymal stem-like cells from human endometrium. Hum Reprod. 2007; 22: 2903-11.

69. Dupas T, Rouaud T, Rouger K, Lieubeau B, Cario-Toumaniantz C, Fontaine-Perus J, et al. Fetal muscle contains different CD34+ cell subsets that distinctly differentiate into adipogenic, angiogenic and myogenic lineages. Stem Cell Res. 2011; 7: 230-43.

70. Dravida S, Pal R, Khanna A, Tipnis SP, Ravindran G, Khan F. The transdifferentiation potential of limbal fibroblast-like cells. Brain Res Dev Brain Res. 2005; 160: 239-51.