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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 Descemets membrane [3, 4]. The corneal epithelium is further divided into three layers: basal, wing, and squames. 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, forniceal 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	Flk-1, CD31 CD144,CD105, CD146, vWF	CD45 CD14
Werner, 2003 [29]	Mouse spleen	PKH-26	ND	CD34, c-Kit, Flk-1 Sac-1	
Bearzi, 2009 [30]	Human myocardium	Flk-1	ND	Flk-1, c-Kit	CD31, vWF

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 dispase 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 anti-inflammation and efficacy of treatment of the diseases characterized by sterile tissue injury and unresolved inflammation [48]. It remains unclear whether such aggregation of NCs mediates quiescence, self-renewal, and progeny production of stem cells.

Cumulative evidence showed that self-renewal of adult stem cells (SC) are regulated in a specialized in vivo microenvironment, termed ''niche'' [49, 50]. 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	Origin	From LESC to LEPC			From LEPC to mature LECs		Mature ECs identifying assay
		Medium Base	Inducer	Markers	Medium Base	Inducer	
Park 2004 [34]	human	hybridoma medium	BMP4 VEGF	Flk-1, CD31	hybridoma medium	BMP4 VEGF	Flk-1, CD31
Ferreira 2007 [31]	Human	EGM-2	FBS	Flk-1, CD34, CD31, CD133	EGM-2	VEGF	CD31, CD34 and Flk-1
Lee 2008 [35]	Murine	hybridoma medium	BMP4	Flk-1, CD31, CD133	Methyl-cellulose medium cytokines	VEGF	Flk-1, CD144
Purpura 2008 [36]	Human	DMEM	BMP4 10ng/mL	Flk-1, CD34	differentiation media	VEGF	CD34 Flk1
Goldman 2009 [33]	Human	DMEM with KO SR	BMP4	Flk-1, CD34, CD31, CD144	EGM-2 cytokine	BMP4 VEGF	Flk-1, CD31, CD144, CD34, and CD133
Noghero 2011 [37]	Murine	N2B27 medium	BMP4	Flk-1, CD31, CD133, CD144	N2B27	hFGF2, VEGF-A165 BMP4	Flk-1, CD31, CD144
Park 2010 [32]	Human	ECSM DMEm/F12 KO serum bFGF	BMP4 PD98059 VEGF bFGF	Flk-1, CD34, CD31, CD133	EGM-2 medium	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			Mature EC Assay
		Medium Base	GFs	Surface	
Goldman 2009 [33]	Human	EGM-2 With cytokine cocktail	VEGF 50ng/ml	24well plate with coated Matrigel	CD31, CD144, CD34
Park 2010 [32]	Human	EGM-2 medium	VEGF bFGF	coated Matrigel dishes	Typical morphologies, express CD31, CD144, vWF, form vascular like structure on Matrigel, and took up acegylated-LDL.

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-β+ bone marrow derived cells has been proved to have the ability to give rise to CFU-F [61], and human endometrium derived MSC are characterized as CD146+/PDGFR-β+, thus PDGFR-β may serve as a marker for MSC precursor cells. Chen et al have prospectively identified and purified vascular pericytes in multiple human organs and shown that these cells are potent mesodermal progenitors that give rise to genuine MSC in culture [62, 63].

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

Author and year	Citations
Corselli 2012 [64]	These novel MSC ancestors, which have been typified as CD34+CD146- cells, can differentiate in culture into CD34-CD146+ pericytes.
Katare 2011 [65]	CD34+ cells, located around the vasa vasorum in the adventitia of arteries and veins, also express typical pericyte markers (NG2, PDGFR-β, 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 [66]	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 [67]	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 [68]	CD146+PDGFR-β+ cells from human endometrium underwent differentiation into adipogenic, osteogenic, myogenic and chondrogenic lineages.

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 ESC 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.

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