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WORD COUNT	2990	TIME SUBMITTED	11-APR-2023 11:40AM
		PAPER ID	98572734

Name of Journal: *World Journal of Stem Cells*

Manuscript NO: 82735

Manuscript Type: MINIREVIEWS

Single-cell RNA sequencing in cornea research: Insights into limbal stem cells and their niche regulation

Sun D *et al*. Review of LSCs

Abstract

The corneal epithelium is composed of stratified squamous epithelial cells on the outer surface of the eye, which acts as a protective barrier and is critical for clear and stable vision. Its continuous renewal or wound healing depends on the proliferation and differentiation of limbal stem cells (LSCs), a cell population that resides at the limbus in a highly regulated niche. Dysfunction of LSCs and their niche can cause failed epithelial wound healing or LSC deficiency, which causes blindness. Nevertheless, compared to stem cells in other tissues little is known about the LSCs and their niche. With the advent of single-cell RNA sequencing, our understanding of LSC characteristics and their microenvironment has grown considerably. In this review, we summarized the current findings from single-cell studies in the field of cornea research and focused on important advancements driven by this technology, including the heterogeneity of the LSC population, novel LSC markers and regulation of the LSC niche, which will provide a reference for clinical issues such as corneal epithelial wound healing, ocular surface reconstruction and interventions for related diseases.

Key Words: Cornea; Limbus stem cells; Single cell transcriptome sequencing; Heterogeneity; Novel markers; Niche regulation

Sun D, Shi WY, Dou SQ. Single-cell RNA sequencing in cornea research: Insights into limbal stem cells and their niche regulation. *World J Stem Cells* 2023; In press

Core Tip: Limbal stem cells (LSCs), a cell population that resides at the limbus in a highly regulated niche. With the advent of single-cell RNA sequencing, our understanding of LSC characteristics and their microenvironment has grown considerably. This review focuses on the current research on single cell sequencing in LSCs. We highlight the heterogeneity of LSCs and present several novel specific markers of LSCs and the role of niche regulation of LSCs.

INTRODUCTION

The cornea is a unique transparent tissue in the human body exposed to the external environment and is the quintessential window for sight[1,2]. Specifically, the corneal epithelium acts as a protective barrier on the ocular surface and is constantly regenerating. This unique property of the corneal epithelium is dependent on self-renewing epithelial stem cells located at the limbus, known as limbal stem cells (LSCs)[3-5]. LSCs reside in the "palisades of Vogt" (also known as limbal epithelial crypts) and are critical for corneal epithelial regeneration and wound healing. LSCs respond to corneal epithelial cell renewal or wound healing by differentiating to produce limbal progenitor cells (LPCs) and transient amplifying cells (TACs), which then migrate to the central corneal basal layer to replenish the corneal epithelium[6-9]. This process was summarized as the "XYZ" hypothesis[10] and explained the balance of cell numbers and homeostasis in the corneal epithelium maintained by LSCs.

Like the stem cells in other tissues, the surrounding microenvironment or limbal niche strictly supports and regulates the functional behaviors of LSCs[11,12]. The limbal niche has unique characteristics and components, including mesenchymal cells, immune cells, melanocytes, vascular cells and neuronal cells, extracellular matrix and signaling molecules (*e.g.*, growth factors and cytokines)[13-16]. Significant pathology involving any component of the limbal niche can lead to regeneration dysfunction of LSCs or even result in LSC deficiency (LSCD), which causes blindness[17,18].

Various studies have identified numerous markers of LSCs but identifying definitive molecular signatures distinguishing LSCs and other corneal epithelial cells is still challenging. The unclear internal heterogeneity of the LSC population can increase the difficulty of efficiently isolating pure LSCs for clinical transplantation. In addition, emerging evidence supports that reconstruction of the limbal niche may treat LSCD. Therefore, understanding LSC function and niche regulation is needed to discover novel therapies for ocular surface disease.

With the development and maturity of sequencing technology, more and more genomic, transcriptomic, epigenetic and proteomic sequencing technologies have been

applied to research in eye tissues[19-22]. In recent years, single-cell RNA sequencing (scRNA-Seq) technology has provided a tool for discovery of new cell types and for dissecting their potential heterogeneity in unprecedented resolution[23-25]. For multicellular organisms, cell heterogeneity is defined by differences in genetic background, differentiation, transcriptome and proteome expression profiles[26]. Compared to other traditional techniques for detecting the average expression of genes in multiple cells, single-cell sequencing can detect differential signals between individual cells, improve the resolution of gene expression research, and explore unknown or rare cell types in tissues[27-29]. A single-cell atlas has been compiled for several ocular tissues, such as the uvea[30], retina[31-34], iris[35,36], sclera[37,38] and human cornea[39,40]. In this review, we summarize the current advances on LSCs derived from single-cell studies to better understand the features and functions of LSCs and the precise cellular and molecular mechanisms of niche regulation. Overall, this review presents key points from recent discoveries to enrich our knowledge on LSC biology and ocular surface homeostasis reconstruction or other clinical problems.

HETEROGENEITY OF THE LSC POPULATION

LSCs are located in the basal layer of the corneal epithelium. As previously mentioned, they are characterized by a high proliferative potential, small size, high nucleoplasmic ratio and slow cell cycle[41,42]. LSCs are scarce, and finding markers to distinguish them from other basal epithelial cells is challenging. In addition, few studies have investigated the heterogeneity and hierarchy of LSCs. However, understanding the heterogeneity of LSCs is important for comprehending the function to effectively isolate them for clinical transplantation.

Dou *et al*[43] performed scRNA-Seq on human limbal tissues and identified four subclusters of stem/progenitor cells after single-cell transcriptome analysis. In this study, the authors annotated eight cell types, including prominent limbal epithelial cells, stromal cells and other rare cell populations. The authors then subclustered limbal epithelial cells and resolved their heterogeneity, including limbal stem/progenitor cells

(LSPCs), limbal suprabasal cells and limbal superficial cells. To further explore the LSC population, the authors then subclustered LSPCs and obtained four subpopulations (Figure 1) including: (1) A subpopulation with the classical LSC marker *TP63*[44]; (2) A subpopulation with high expression of *CCL20*, which is a chemokine that can induce cell migration and proliferation[45]; (3) A subpopulation with specific expression of *Gpha2*, a marker recently identified in quiescent LSCs (qLSCs) from humans and mice[46,47]; and (4) A subpopulation with high expression of *KRT6B*, which is associated with rapid keratinocyte division and contributes to inhibiting the migration of mitotic cell populations from the basal layer[48]. The authors then investigated the differences in stemness and differentiation status and observed that *TP63*$^+$ and *CCL20*$^+$ cells presented a high stemness state, whereas *Gpha2*$^+$ and *KRT6B*$^+$ showed a high differentiation state.

Another study by Li *et al*[49] annotated five subtypes from the limbal basal epithelium of the human cornea. They characterized terminally differentiated cells (TDCs), post-mitotic cells, TACs, LPCs and LSCs. Furthermore, the authors discovered that these five subtypes represented the major stages and trajectories of human LSC proliferation and differentiation (from LSCs, LPCs, TACs and post-mitotic cells to TDCs), and they were spatially situated in different regions from the limbus to the central cornea. In TDCs, corneal epithelium-specific differentiation markers and keratinocyte keratinization markers were expressed at the highest levels, while the LSC differentiation markers had the lowest expression.

LSCs in mice are also heterogeneous and behave differently than human LSCs. Altshuler *et al*[46] combined scRNA-Seq and quantitative lineage tracing for in-depth analysis of mouse limbal epithelium. The authors revealed the presence of two distinct subpopulations of mouse LSCs that were in separate and well-defined spatial locations called the "inner" and "outer" limbus (Figure 2). The inner corneal limbus contains active LSCs, which maintain the homeostasis of the corneal epithelium. The outer limbus contains qLSCs that have a significantly lower rate of division and are involved in wound healing and border formation. Spectral tracking experiments displayed that qLSCs can quickly exit the dormant state and enter the cell cycle in response to injury,

suggesting that qLSCs are a reservoir for tissue regeneration. In addition, their circumferentially extended clonal growth model and continuous localization on the border highly indicates that these cells play a crucial role in border maintenance. Notably, this finding was also confirmed by a study utilizing the two-photon live imaging approach[50]. Collectively, LSCs are highly heterogeneous in both humans and mice, unlike stem cells in other tissues. Further studies are needed to investigate the self-renewal and differentiation mechanisms of LSCs.

NOVEL MARKERS FOR LSCS

Since 1989, when LSCs were discovered[4], a series of markers have been found to identify these cells, such as Tp63, Krt3, Krt12. However, the marker pattern typically labels the broad limbal basal cell population. Accurately distinguishing LSCs from other epithelial cells is still challenging and is still an active area of research. Altshuler *et al*[46] discovered a novel set of markers to accurately identify LSCs. They applied *in situ* hybridization probes for *Krt4* and *Krt12* to label mouse conjunctival and corneal basal and suprabasal cells, respectively. *Gpha2*$^+$ signaling could obviously demarcate the outer LSCs (also known as qLSCs), while the inner LSCs (also known as active LSCs) were labeled as *Atf3*$^+$. Then, they used immunofluorescence staining to confirm that the outer limbal epithelial basal cells were *Krt15*$^+$/*Ifitm3*$^+$/*Cd63*$^+$, and the inner limbal epithelial basal cells were *Atf3*$^+$/*Mt1-2*$^+$. Next, the authors explored the correlation between mouse and human LSC markers. Immunofluorescence images revealed that *KRT15*$^+$, *Ifitm3*$^+$ and *Gpha2*$^+$ were expressed in human limbus epithelial basal cells. *Ifitm3*$^+$ was found to be restricted to cellular vesicles in the cytoplasm of undifferentiated limbal cells, which was consistent with a previous study's findings[51]. *Ifitm3*$^+$ knockdown led to a differentiation phenotype and a reduced colony-forming capacity. These experiments suggest that *Ifitm3*$^+$ and *Gpha2*$^+$ can be used to identify LSCs, and *Ifitm3*$^+$ mediates the undifferentiated state.

Gpha2$^+$ has been frequently studied in human LSCs. Dou *et al*[43] explored the four subclusters of LSPCs, which were identified by TP63, CCL20, GPHA2 and KRT6B.

Collin *et al*[47] identified several novel genes, one of which was *Gpha2*$^+$, using an unbiased approach to recognize marker genes that were highly expressed in human LSCs relative to other corneal epithelial cells. High and specific expression of *Gpha2*$^+$ was observed in the limbus crypts, which was consistent with the findings of Altshuler *et al*[46]. Moreover, the authors used RNA interference (RNAi) to downregulate GPHA2 and showed a significant reduction in cell proliferation and differentiation efficiency, indicating an important role of GPHA2 in maintaining the undifferentiated state of human LSCs. The authors also performed flow activated cell sorting analysis with colony forming efficiency assays to confirm the RNAi data.

Other LSC markers have also been identified. Li *et al*[49] identified *TSPAN7*$^+$ and *SOX17*$^+$ cells distributed in a scattered pattern in human limbus epithelium basal cells. The authors established an *in vitro* model of epithelial cells and discovered *TSPAN7*$^+$ and *SOX17*$^+$ were not strongly expressed in human limbal epithelial cells. However, mRNA and protein expression levels were significantly activated after injury, especially during cell migration and growth. The authors also utilized RNAi to downregulate *TSPAN7*$^+$ and *SOX17*$^+$ and observed inhibited cell proliferation and significantly delayed epithelial regeneration during wound healing. Overall, the discovery of novel markers of LSCs (Table 1) can help us to better distinguish LSCs from other cells to further understand the function and state of LSCs and provide a more effective strategy for the isolation, culture and clinical application of LSCs.

NICHE REGULATION OF THE LSCS AT THE LIMBUS

LSC proliferation, migration and differentiation are inseparable from the regulation of the limbal niche microenvironment. The stem cell niche is the local microenvironment directly promoting or protecting stem cell populations[52-54]. The LSC niche provides a sheltered environment that protects LSCs from stimulation[55-58]. If the LSC niche is involved in pathological damage, then LSC dysfunction can occur. Therefore, the study of the LSC niche is essential.

Collin *et al*[47] investigated the interaction between LSCs and the limbal niche by single-cell analysis. The authors combined scRNA-Seq and ATAC-Seq and performed CellPhoneDB analysis[59]. They identified multiple significant interactions between human LSCs and regulatory factors of immune cells such as proinflammatory cytokines [tumor necrosis factor, interleukin (IL)-1β, IL-6, IL-17A, interferon γ, and oncostatin M], proinflammatory cell surface receptor (triggering receptor expressed on myeloid cells 1), proinflammatory cytokine expression (adaptor complexes 1) and regulators of inflammatory responses (nuclear factor kappa B, RELA, colony-stimulating factor 2, phosphoinositide 3-kinase, extracellular signal-regulated kinase 1/2, and F2). The authors verified that limbal epithelial cells were significantly reduced in cell culture medium containing tumor necrosis factor-α and IL-1β. This suggested that proinflammatory cytokines produced by immune cells were involved in the apoptosis of limbal epithelial cells[60], thus mimicking the central corneal defect and stimulating the proliferation of LSCs[61]. This was also consistent with other reports showing that the addition of proinflammatory factors to limbal epithelial cell cultures can directly affect the expression of LSC markers and their colony forming efficiency capacity[60,62-64].

Dou *et al*[43] systematically explored intercellular communication between LSPCs and other cell populations based on ligand-receptor analysis. By correlating the corresponding receptor-ligands in human LSPCs and their niche cells, the authors observed that LSPCs were regulated by the limbal niche as well as by other cells in the limbal niche. The NOTCH signaling pathway was also involved in cell-cell interaction between LSPCs and their niche cells. NOTCH1-4 receptors were expressed in LSPCs, and their relevant ligands were primarily identified in niche cells, such as Schwann cells, stromal cells, pericytes and LSPCs. Likewise, the WNT7A, WNT7B and WNT5A ligands, which participate in the Wnt/β-catenin signaling pathway, were detected on LSPCs. Their corresponding receptors were primarily detected on limbal epithelial cells, stromal cells, immune cells, Schwann cells and LSPCs. The presence of multiple chemokines, such as CCL4, CCL4L2, IL-1β and IL-24, on LSPCs and their paired receptors indicated that immune cell interactions may potentially regulate LSPCs.

Altshuler *et al*[46] revealed that T cells acted as niche cells and served its function in the maintenance of quiescence, epithelial thickness control and wound healing. By studying the limbus of the immunodeficiency (SCID) and non-obese diabetic SCID mice, which are unable to make mature T and B lymphocytes, it was observed that the GPHA2 protein was substantially decreased to almost undetectable levels. In contrast, the expression of *Ifitm*$^{3+}$ did not rely on the existence of immune cells, implying that it was regulated by other niche cells. When T cells were inhibited by topical application of the corticosteroid dexamethasone, LSCs showed a dramatic reduction in *Cd63* and *Gpha2* expression levels and increased cell proliferation, demonstrating that T cells played a crucial role in regulating qLSCs. Finally, corneal epithelial debridement followed by epithelial closure by fluorescein dye infiltration revealed delayed epithelial wound healing in mice lacking T cells.

In addition, other niche cells were determined to be important for the microenvironment regulation of LSCs. Oxidative stress can lead to a variety of eye diseases, such as keratitis, cataracts and retinal diseases, which are subject to varying degrees of oxidative damage[65,66]. Recently, studies found that melanocytes in the limbal niche (as antioxidant systems) protected LSPCs from UV-induced oxidative damage and reduced oxidative stress through the transfer of melanosomes[67,68]. Moreover, by ligand analysis, Dou *et al*[43] identified the intercellular communication between melanocytes and LSCs. NAMPT was highly expressed in melanocytes as a ligand and had been reported to act as a critical switch in melanoma cells. CD44 acted as a receptor and was also highly enriched in melanocytes.

Vascular endothelial cells are also one of the important niche cells of LSCs. It has been reported that vascular endothelial cells were highly correlated with the classic Wnt signaling pathway involved in the regulation of the corneal limbal niche[69,70]. Furthermore, Dou *et al*[43] performed a differential expression analysis with the integration of the scRNA-Seq dataset from the limbus and the skin and observed that the vascular endothelial cells from the limbus highly expressed antivascular factors compared to that from the skin, consistent with characteristics of corneal angiogenic

privilege. Above all, these studies have shown that the regulation of the LSC niche (Figure 3) occupies a key role in the growth, development, proliferation and differentiation of LSCs.

CONCLUSION

The first Drop-Seq experiments were performed on mouse retina in 2015[23]. Since this revolutionary experiment, single-cell sequencing technology has been widely used in many fields, including ophthalmology, and gene expression has been studied at an unprecedented level of resolution in multiple ocular tissues. Corneal transparency is essential for normal vision; thus, comprehension of the mechanisms related to corneal wound healing and regeneration is crucial for the treatment of patients suffering from corneal disease. Currently, corneal epithelial regeneration is a relatively satisfactory approach and has the potential to treat corneal superficial scars. However, for multiple corneal basal scars or endothelial disease, corneal transplantation remains the only option to restore clear vision[71-73]. Unfortunately, corneal clouding remains one of the leading causes of blindness worldwide due to the lack of corneal donor tissue or the limited availability of corneal surgery[74,75]. Although most studies support corneal regeneration through LSC therapies[76,77], the study of LSCs is particularly important.

This review focused on the current research on single-cell sequencing in LSCs. We highlighted the heterogeneity of LSCs and presented several novel specific markers of LSCs and the role of niche regulation of LSCs. LSCs can be identified in both humans and mice, and several markers, such as GHPA2 and IFITM3, can be highly and specifically expressed on LSCs. Moreover, both T cell regulation in mice studied by Altshuler *et al*[46] and immune cell regulation in humans studied by Collin *et al*[47] and Dou *et al*[43] suggest that niche regulation is of vital importance for LSCs.

Future research can still benefit from RNA-Seq technology as it can aid in acquisition of further knowledge on the functions and characteristics of LSCs, including in the discovery of more novel highly specific expression markers and more niche regulated components that can promote or inhibit the proliferation and differentiation of LSCs.

These discoveries should be translated into better prevention and treatment strategies to treat blindness and improve the clinical prognosis of patients with LSCD and other LSC-related diseases.

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