Point-to-point response

Reviewer 1:

Language in the MS needs better polished. For example, page 3, 2nd last para, the sentence "…distinguishing LSCs and other epithelial cells is still challenging" needs a better expression. Some symbols did not show up properly, leading the reviewer unable to comment on their accuracy.

Response: We greatly appreciate the detailed feedback from this reviewer. We attach great importance to these comments and suggestions, which are valuable and help to improve this article. In this revision, we have made sufficient revisions based on your comments. We hope that the revised manuscript will satisfy you.

Reviewer 2:

The review article by Sun et al. and titled "Single-cell RNA sequencing in cornea research: Insights into limbal stem cells and their niche regulation", aims to highlight the recent developments related to the applications of scRNA sequencing in understanding the limbal niche, niche cell population diversity, their unique and novel marker based identities and the regulation of corneal tissue homeostasis. It is a well written short review article that nicely covered the recent work that employed scRNA seq to characterize limbal tissue cell types and also the validations done to establish them as unique markers for different cell types. This manuscript can be improved further based on the comments below:

Response: Thank you very much for your enthusiasm and affirmation of our work. We also thank you for your clear and detailed feedback, which has greatly improved the quality of this manuscript, and hope that the interpretation has adequately addressed all your concerns. In this revision, we will discuss each of your comments and our answers accordingly, respectively.

1. A brief description of the scRNA method and its relative advantages over other methods can be added to highlight the significance and usefulness of the method in understanding limbal biology.

Response: We think this is an excellent suggestion. For this section, we added a brief description in **Introduction**:" For multicellular organisms, there are differences between cells, that is, cell heterogeneity, such as genetic background, differentiation, transcriptome, and proteome expression profiles[1]. 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[2-4] ."

2. While describing the newer study findings and markers identified, the authors can also highlight the well-known markers described and validated so far, to consolidate the available information for the readers.

Response: Thank you for this suggestion. In the Table 1 of this version, we have compiled and summarized the specific LSC markers identified in humans and mice, with references attached. And we added some known classical marker to the "Novel markers for LSCs" section.

2. The concluding statement is too complex and can be split into simple sentences.

Response: Thank you very much for your advice, in this version, we have modified and adjusted the conclusion section accordingly.

Reviewer 3:

This is an excellent review on the field of the insights into limbal stem cells and their niche regulation. The manuscript is very well written and particularly useful to undestanding the progress made in this field in the last years. Very important for physicians dealing with corneal problems and corneal transplantation. Important and updated references.

Response: Thank you very much for your time involved in reviewing the manuscript and your very encouraging comments on the merits. If there are any other modifications we could make, we would like very much to modify them and we really appreciate your help. Thanks again for your positive comments of the quality of the manuscript.

Reviewer 4:

Sun et al. realized a very interesting minireview describing the "Single-cell RNA sequencing in cornea research: Insights into limbal stem cells and their niche regulation". I consider the manuscript very interesting but, at the same time, I suggest several revisions needed to improve the reliability and the completeness of the paper:

Response: We greatly appreciate your professional review of our manuscript. These opinions are all valuable and help to improve the quality of our manuscript. We revised the manuscript and added additional data to make our results convincing. The detailed point-by-point responses are listed below.

1. The "Novel markers" and "Niche regulation" sections should be more updated and improved. I suggest adding data related to the involvement of oxidative stress, also focusing on vascular components, in relationship to the eye component of the pathology. The recent PMID: 32877751, PMID: 30523548, PMID: 36490268 and PMID: 36290689 could represent a substrate able to enforce the role of considered cellular mechanisms.

Response: Thank you for pointing this out. We carefully investigated the relevant literature, and in this version, we cited PMID: 36490268 in **Introduction**. In the **niche regulation**, we increased oxidative stress and vascular endothelial cells by citing PMID: 32877751, PMID: 30523548 and PMID: 36290689:"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[5,6]. 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[7,8]. Moreover, by ligand analysis, Dou *et al*[9] 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[10,11]. Furthermore, Dou *et al*[9] 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."

2. Finally, manuscript requires important English revisions and typos correction.

Response: Thanks for your kind reminding, and we apologize for the inaccurate English revisions and typos. In this new submission, we have carefully and thoroughly proofread the manuscript to correct all the English revisions and typos.

Reviewer 5:

Abstract: Please note that whole outer surface of eye is not covered by Corneal Epithelium. Kindly modify accordingly. Conclusion: The authors need to provide more insight regarding future prospect of LSC single-cell RNA sequencing in Ocular Surface Reconstruction.

Response: Thanks for these suggestions.We apologize for the description in **Abstract**. In this revised version, we corrected it as "The corneal epithelium is composed of stratified squamous epithelial cells on the outer surface of the eye". About **Conclusion**, we added some insight: Future research still needs to use RNA-seq technology to further understand the functions and characteristics of LSCs, such as discovering 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.

Reference

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[4] **Svensson V, Vento-Tormo R, Teichmann SA**. Exponential scaling of single-cell RNA-seq in the past decade. *Nat Protoc*. 2018;**13(4)**:599-604. [PMID: 29494575 DOI:10.1038/nprot.2017.149]

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