August 16th, 2021

Re: JoVE62820

We are thankful to the editor and the reviewers for their insightful comments and suggestions that improved the quality of our manuscript. We have performed additional experiments, revised the manuscript, and answered all concerns raised by the reviewers. We have changed the manuscript format according to the Journal guideline.

The following are our point-by-point responses to the editor and reviewer's comments.

Editorial comments

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We sent our manuscript for academic proofreading for English-language improvement and carefully corrected English errors and writing styles in the revised manuscript.

2. Please provide an email address for each author.

We have provided the email addresses of all authors.

3. Please ensure that the long Abstract is within 150-300-word limit and clearly states the goal of the protocol.

We revised the abstract, and it contained 166 words.

4. Please ensure that in text citations follow the numbering order. So, 1 will be before 2 and so on. Please organize the references accordingly.

We organized the references accordingly.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Thermo Fisher Scientific, EpiLife, Defined Trypsin Inhibitor, Matsunami Glass,

Triton-X (Wako), (BD Biosciences, 553745), (BioLegend, 905301), Nikon A1, Adobe Photoshop software, Sigma, etc.

We removed all trademark symbols and company names and replaced them with generic terms instead.

6. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

The ethical statement was added as the first sentence in the Protocol section (Line 91-92).

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

We revised the manuscript accordingly.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We confirmed that our protocol contains all the content and detailed description that we want to include in the video.

9. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

According to Jove's requirements, we adjusted the format, including font, paragraph indentation, and line spacing.

10. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies

the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We highlighted the necessary sections in yellow (Protocol steps 1 to 4) in the revised manuscript for filmable content.

11. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

We moved all Figure Legends at the end of the Representative Results in the revised manuscript.

12. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the techniques.

We followed the Jove's instructions and added some citations in the Discussion in the revised manuscript.

Reviewers' comments
Reviewer #1:
Manuscript Summary:
The authors described a detailed protocol to isolate and culture epithelial cells from adult mouse hard palate. The manuscript is well written. However, similar techniques have been used in other studies to isolate epithelia cells from other oral tissues.

We thank the reviewer for their valuable comments, suggestions on references, and careful reading of our manuscript. As the reviewer pointed out, protocols from other mouse oral tissues (e.g., gingiva and tongue) exist, but this is the first one using the palate. We believe our protocol is valuable in the field.

Minor Concerns:
1: Line 77, please cite the following papers:

Simões A, Chen L, Chen Z, Zhao Y, Gao S, Marucha PT, Dai Y, DiPietro LA, Zhou X. Differential microRNA profile underlies the divergent healing responses in skin and oral mucosal wounds. Sci Rep. 2019 May 9;9(1):7160. doi: 10.1038/s41598-019-43682-w. PMID: 31073224; PMCID: PMC6509259.

Turabelidze A, Guo S, Chung AY, Chen L, Dai Y, Marucha PT, DiPietro LA. Intrinsic differences between oral and skin keratinocytes. PLoS One. 2014 Sep 8;9(9):e101480. doi: 10.1371/journal.pone.0101480. PMID: 25198578; PMCID: PMC4157746.

Chen L, Gajendrareddy PK, DiPietro LA. Differential expression of HIF-1α in skin and mucosal wounds. J Dent Res. 2012 Sep;91(9):871-6. doi: 10.1177/0022034512454435. Epub 2012 Jul 19. PMID: 22821237; PMCID: PMC3420394.

Chen L, Arbieva ZH, Guo S, Marucha PT, Mustoe TA, DiPietro LA. Positional differences in the wound transcriptome of skin and oral mucosa. BMC Genomics. 2010 Aug 12;11:471. doi: 10.1186/1471-2164 11-471. PMID: 20704739; PMCID:PMC3091667.

We appreciate your valuable suggestions. These references were added to the introduction part of the manuscript (Page 3, Line 84).

2: Line 120: "dermis region" should be called "lamina propria"

Thank you for pointing out this critical point. We revised the manuscript accordingly.

3: Line 135 and 136 Description of "Transfer the remaining tissue to 4 mL of complete medium in another 60-mm dish. Gently scrape the epithelial side again" is not clear. "remaining tissue" should be scraped off epithelial layer.

We agreed that our explanation was not clear. After first scraping in the trypsin inhibitor solution, we transfer the tissue in another 60-mm dish containing 4 mL of complete medium and scrape again. The purpose of the second scraping is to collect as many keratinocytes as possible. Thus, we revised our text to explain this part more clearly (Page 5, Line 231-237).

Reviewer #2:
Manuscript Summary:
The manuscript is to describe a protocol to isolate and culture-expand oral primary

keratinocytes from the palatal tissues of adult mice, and it will be useful for research application by using mouse cells to study oral diseases.

We thank the reviewer for their critical comments. According to the reviewer's suggestions, we have performed additional experiments, revised the manuscript, and answered all concerns raised by the reviewer.

Major Concerns:

(1) Contamination of fibroblast is the main problem. What percentage of fibroblast contamination? Any evidence for the contamination? For example by staining vimentin to quantify the contamination. And please give any solution to avoid this contamination during the isolation?

We agreed that fibroblast contamination is a crucial issue. However, with our current protocol, this contamination rate is very low (less than 5%) and rarely happens with experienced people. Our protocol uses commercial culture media, which contains fibroblast growth inhibitors such as hydrocortisone, triiodothyronine.

To test the fibroblast contamination in keratinocyte culture, we performed additional immunostaining using a fibroblast marker PRGFRα based on the reviewer's suggestion. We confirmed that no fibroblast was observed in both early and late passage cultures (new Figure. 3I-L).

(2) The authors claimed that the epidermal cells they isolated and expanded are stem cell like cells, any evidence? Colony formation assay? Expression of Stem cell markers?

We thank the reviewer for the critical comment. To assess the stem cell-like characteristics of primary keratinocytes, we performed staining using p63 as a reported stem cell marker. According to our results, p63 was highly and uniformly expressed in both early passage (passage 4) and late passage (passage 7) (new Figure 3C, D), indicating their stem cell-like status.

(4) How many passages of the epidermal cells can be maintained without significant differentiation? Could author provide some staining of differentiation markers such as keratin 1 or 10 or loricrin in passaged cells.

As the reviewer suggested, we performed immunostaining of K13 that was previously identified as an oral epithelium differentiation marker. We confirmed that K13 expression was negative at least until passage 7, compared to the differentiation condition with High Calcium treatment (Figure 3F-H).

(3) Is any reason to use 0.04% trypsin? Not 0.05% trypsin which normally we obtained.

Our protocol follows the previously established protocol in human oral keratinocytes, in which they used 0.04% trypsin (Izumi *et al*., *J Dent Res* 2007). We adopted 0.04% trypsin because this condition is also effective for isolating oral keratinocytes in mice.

(4) Is there any difference between the hard palate and soft palate for isolation of epidermal cells? I am not very sure which one used in the protocol.

I apologize for the confusing explanation. The hard palate and soft palate are not separated in our protocol because the tissues are too small. Therefore, we have a mixture of cells from both the hard and soft palates. Since the hard palate is larger in size, we believe that most isolated keratinocytes are derived from the hard palate.

(5) What passage of cells was used for analysis in Figure 3?

Thank you for pointing this out. In the previous Figure 3, we used cells from passage 4. In the revised Figure, early passage (passage 4) and late passage (passage 7) are used, and the passage numbers are indicated in the figure legend and text.

(6) The resolution of figure images is low, please improve. And please carefully edit the language

We think the resolution of Figure images was high in the first submission, but due to the online system, it is possible that lower resolution images were passed on to the reviewer. In the revision, we carefully rechecked the resolution of all Figures. The manuscript has been proofread in English.

Minor Concerns:

Line 99: Please provide the ethical statement for animal study

The ethical statement was added as the first sentence in the Protocol section (Line 91-92).

Line 104: Is 10% povidone solution: "povidone-iodine"?

In the revised manuscript, we have corrected the 10% povidone solution to povidone-iodine.

Line 110: Please indicate the time frame that the tissue can be keep on ice for next. step?

The palatal tissues could be kept on ice for up to four hours. We added this explanation in the revised manuscript (page 4, line 144).

Line 129: It is not very clear how to scrape the epithelial layer? Since this step is very critical, please describe more details how to efficiently take off the epithelial layer without remove of dermal part. How to collect the removed epidermal layer? By using what solution (PBS) to collect the epidermal layer for filtration? Did it require to disperse the removed epidermal layer to get

Thank you for the reviewer's insightful comments. We think our previous explanation was not clear. We carefully rewrote the part of scraping and collecting cells from the tissue (page 5, line 231-239) as follows:

1. Using one pair of blunt forceps, grasp tissue from trypsin solution (in the 35-mm dish), and transfer to trypsin inhibitor solution in a 60-mm dish (4 mL per dish) with the epithelial surface facing up.
2. Using forceps to hold on the edge of the palate, gently scrape the epithelial layer off the underlying lamina propria using a scalpel blade (#15).
3. To collect maximum epithelial cells from tissues, transfer the tissue in another 60-mm dish with 4 mL complete medium and repeat the scraping step.

NOTE: Make sure to try not to scrape tissue by the blade's tip; use the edge part instead. Scraping time is approximately 5-10mins per tissue.

4. Place a sterile 100-µm cell strainer on top of a 50-mL conical tube.
5. Using a sterile pipet, transfer 2 mL of trypsin solution from (1) into the strainer to wet its surface.
6. Using a pipet, mix the cell suspension in the 60-mm dish (2) (3) a few times and filter cells through the 100-µm cell strainer prepared in (4) (5).

Line 126-133: these steps are not very clear to me, was the digestion solution with scrapping collected for epidermal cells as well?, please clarify.

We apologize for our unclear explanation. As indicated in the protocol above, tissues incubated in trypsin are transferred to trypsin inhibitor solution on the second day to stop the reaction (protocol, step 4-1). 2 mL of the remaining trypsin solution is used to wet the surface of a 100-µm cell strainer (protocol, step 4-5). The final cell suspension will contain 2 mL of trypsin, 4 mL of trypsin inhibitor solution, and 4 mL complete medium.

Line 203: Please specify how long was the cells cultured before fixation for immunofluorescence staining.

Thank you for your careful reading. We usually seed about $0.5x10^6$ cells per coverslip and culture for 2 days before staining. We added this in the revised manuscript.

Reviewer #3:
This study by Ngo et al. gives clear understanding of the protocol for isolation and culture of primary oral keratinocytes from adult mice. The manuscript is very well written and the protocol followed is innovative and up to date. This study surely will have some value in translational research such as in vitro disease modelling, preclinical screening and even establishing a stable cell line. Getting pure population of primary keratinocytes is a main hurdle and due to many studies produce false results. This protocol will certainly help in crossing these barriers and produce high quality research in the future. Taking into consideration some minor concerns will help improving the quality of the manuscript:

We appreciate the reviewer's positive comments and suggestions. We carefully revised our manuscript accordingly.

1. In the table Table 1 'sell' needs to be changed to 'cell'.

Thank you for pointing this out. We revised the manuscript accordingly.

2. In the Table 2 'Popiyodon' need to be changed to 'povidone'.

We revised the manuscript accordingly.

3. In the future studies, along with immunofluorescent staining, I will strongly recommend RT-qPCR and Western blot analyses to compare heterogeneous population and purified keratinocytes for a conclusive remark.

We thank the reviewer for their valuable comments. We also believe that the experiments proposed by the reviewer will help us to further understand the cellular heterogeneity and purity of primary oral keratinocytes in the future.