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TITLE:

Isolation and Culture of Primary Oral Keratinocytes from the Adult Mouse Palate

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KEYWORDS:

Epithelial stem cells, oral epithelium, oral keratinocyte, mouse keratinocyte, primary cell culture, palate

SUMMARY:

The present protocol describes the isolation and culture of oral keratinocytes derived from the adult mouse palate. An evaluation method using immunostaining is also reported.

ABSTRACT:

For years, most studies involving keratinocytes have been conducted using human and mouse skin epidermal keratinocytes. Recently, oral keratinocytes have attracted attention because of their unique function and characteristics. They maintain the homeostasis of the oral epithelium

and serve as resources for applications in regenerative therapies. However, *in vitro* studies that use oral primary keratinocytes from adult mice have been limited due to the lack of an efficient and well-established culture protocol. Here, oral primary keratinocytes were isolated from the palate tissues of adult mice and cultured in a commercial low-calcium medium supplemented with a chelexed-serum. Under these conditions, keratinocytes were maintained in a proliferative or stem cell-like state, and their differentiation was inhibited even after increased passages. Marker expression analysis showed that the cultured oral keratinocytes expressed the basal markers p63, K14, and α6-integrin and were negative for the differentiation marker K13 and the fibroblast marker PDGFRα. This method produced viable and culturable cells suitable for downstream applications in the study of oral epithelial stem cell functions *in vitro*.

INTRODUCTION:

The oral epithelium serves as a first barrier in protecting the body from environmental stresses, including chemical or physical damage and bacterial and viral infections[1,2]. The oral mucosa comprises an outer layer of stratified squamous epithelium that consists of keratinocytes and underlying connective tissue called the lamina propria, which mainly consists of fibroblasts and the extracellular matrix. The mouse oral mucosa can be broadly divided into three subtypes: masticatory (hard palate and gingiva), specialized (dorsal tongue), and lining (buccal mucosa, ventral tongue, soft palate, lips) mucosa[2,3] (**Figure 1A**). The oral epithelium is keratinized in the masticatory and specialized mucosa and non-keratinized in the lining mucosa. Despite its anatomical location, the oral epithelium is similar to the skin epidermis in that it consists of tightly packed epithelial cells with varying degrees of differentiation: basal layers containing undifferentiated cells; spinous, granular, and cornified layers that form keratinized epithelium, or intermediate and superficial layers that form non-keratinized epithelium[4]. Transgenic mouse models have facilitated the study of oral epithelial stem cells' cellular and molecular features in the palate, buccal mucosa, tongue, and gingiva[5–11]. However, most of these studies primarily used *in vivo* mouse experiments. Cell culture systems were not typically employed owing to a lack of established and efficient protocols.

An *in vitro* culture system can be used for the molecular and biochemical analysis of stem cell regulators, cell-based assays, and drug screening. Currently, protocols for the culture of primary keratinocytes of the skin epidermis have been developed, in which basal keratinocytes can be successfully isolated and cultured for clinical and research purposes[12–15]. In 1980, Hennings et al. showed that a low calcium concentration (< 0.09 mM Ca^{2+}) in the culture medium facilitated proliferation and maintained cells in an undifferentiated state. A higher level of calcium promoted cell differentiation and reduced proliferation[16]. Subsequently, culture methodologies for neonatal and adult murine epidermal keratinocytes have been established and widely applied to numerous mouse models with different genetic backgrounds for *in vitro* studies[17–19]. Although skin and oral epithelia share common characteristics, they also show intrinsic differences, e.g., in their keratinization status, turnover rate, gene expression, and wound healing ability[3,11,20–26].

Although human oral keratinocyte culture has been successfully performed[27–29], publications on mouse oral keratinocyte culture[30–32] are limited due to the small size of the target tissue and the

distinct characteristics of the cells compared to skin epidermal keratinocytes. This protocol describes the isolation and long-term culture techniques of mouse primary oral keratinocytes.

PROTOCOL:

All animal experiments were performed according to the Institutional Animal Experiment Committee guidelines at Kumamoto University and the University of Tsukuba.

1. Preparation of reagents and culture media

1.1. Prepare 40 mL of keratinocyte culture medium containing 60 µM of calcium and 600 µL of antibiotic-antimycotic solution. Prepare 20 mL of 0.025% trypsin and 400 µL of antibiotic-antimycotic solution.

1.1.1. Thaw the trypsin inhibition solution at room temperature and keep it at 4 °C until use in step 4.1.

NOTE: The isolation reagent is prepared for the tissue isolation of five mice.

1.2. To prepare the culture media, take 500 mL of medium and add 5 mL of a growth supplement solution (see **Table of Materials**) (hereafter referred to as complete medium) and 20% calcium-depleted chelexed-fetal bovine serum (FBS)[33] (hereafter referred to as chelexed-FBS).

2. Dissection of palate tissue from adult mouse

2.1. Sacrifice an adult C57BL/6J mouse (either male or female) by cervical dislocation in compliance with the facility's regulations relating to animal welfare.

2.1.1. Remove the hair around the mouth with a shaver. Using scissors, cut from the cheek toward the jaw, on both sides.

NOTE: The mouse needs to be anesthetized before sacrifice. An anesthetic mixture of medetomidine, midazolam, and butorphanol is used (see **Table of Materials**).

2.2. Use forceps to open the mouth wide and absorb any blood using a cotton swab. To disinfect the palate, wipe the inside of the mouth with a cotton swab containing 10% povidone-iodine.

2.3. To harvest the mouse palate, first, use a surgical scalpel blade to make a full-thickness marginal incision along the palate side of the maxillary teeth. Then, carefully dissect the entire palate using a raspatorium.

NOTE: A raspatorium is a tool used to elevate a mucoperiosteal flap (see **Table of Materials**) (**Figure 1B**).

2.4. Quickly transfer the palate tissue to a 15 mL tube containing 4 mL of complete medium + antibiotic-antimycotic solution. Keep the tissues on ice until ready for incubation.

NOTE: For collection from multiple mice, palate tissues may be kept on ice for up to 4 h.

3. Pretreatment of palate tissue

3.1. In a laminar flow hood, transfer tissues to a 60 mm dish containing 4 mL of complete medium + antibiotic-antimycotic solution.

3.2. Using short, blunt forceps and a scalpel blade, gently remove any blood from the tissues. Wash tissues 10 times in complete medium.

3.3. Transfer tissues to a 35 mm dish containing 4 mL of 0.025% trypsin + antibiotic-antimycotic solution, with the epithelial surface facing down.

NOTE: The epithelial surface, which curves inward, should be soaked in the trypsin solution; the lamina propria should face up. The tissue should be flattened as much as possible to be incubated entirely in the trypsin solution.

3.4. Incubate tissues in 0.025% trypsin for ~16 h at room temperature in the culture hood.

4. Collection and culture of primary cells

4.1. Using one pair of blunt forceps, remove 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.

4.2. Using forceps to hold onto the edge of the palate, gently scrape the epithelial layer off the underlying lamina propria using a scalpel blade.

NOTE: Connective tissue is not digested by trypsin, so it does not peel off during scraping.

4.3. To collect the maximum amount of epithelial cells from tissues, transfer the tissue into another 60 mm dish with 4 mL complete medium and repeat the scraping step.

NOTE: Make sure to avoid scraping the tissue with the blade's tip; use the blade's edge instead. Scraping is performed for ~5–10 min per tissue.

4.4. Place a sterile 100 µm cell strainer on the top of a 50 mL conical tube.

4.5. Using a sterile pipette, transfer 2 mL of trypsin solution (from step 4.1) into the strainer to wet its surface.

4.6. Using a pipette, mix the cell suspension in the 60 mm dish (from steps 4.2–4.3) a few times and filter cells through the 100 µm cell strainer prepared in steps 4.4–4.5.

4.7. Count the number of cells using a hemocytometer. Prepare 15 µL of trypan blue solution and add 15 µL of cell suspension (step 4.6). Transfer 10 µL of the cell-trypan blue mix to a hemocytometer and count the number of cells.

NOTE: One piece of mouse palate can yield up to 1 million cells.

4.8. While counting, centrifuge the tube (from step 4.6) at 100 x *g* for 5 min at room temperature.

4.9. Aspirate the supernatant with a pipette. Add 2 mL of complete medium + chelexed-FBS to the tube. Resuspend the cell pellet by triturating several times using a 5 mL pipette.

4.10. Plate 2–5 x 10^5 cells from one mouse into one well of a 24-well plate pre-coated with Collagen Type I (see **Table of Materials**).

4.11. Incubate the cells at 37 °C for 2 days without changing the medium.

4.12. Two days after seeding, replace half of the culture medium with the complete medium + chelexed-FBS. Check the cell morphology under the microscope. Feed cells with complete medium + chelexed-FBS every 2 days.

NOTE: Following seeding, cells of different sizes will be observed. Approximately 3–5 days after seeding, keratinocytes with a cobblestone morphology (**Figure 2**) can be observed. It will take 1–2 weeks of culture before the first passage can be conducted and a subsequent 1–2 weeks is necessary before the cells are ready for the second and third passages. After that, cells will grow faster and may be prepared for cryopreservation. Cells can be re-plated to one well (of a 24-well plate) and a 6-well plate in the first and second passage, respectively. The subsequent passage will be dependent on the cell growth and density. A cell split ratio of 1:2 or 1:3 can be used after the third passage.

5. Keratinocyte passage

5.1. Collect 2 mL of the supernatant from the culture dish and dispense into a 15 mL conical tube (on ice). Wash the cells with sterile 1x PBS twice.

NOTE: When the cells reach approximately 70%–80% confluency, they are ready for passage.

5.2. Add 1 mL of 0.05% trypsin-EDTA to the dish.

5.3. Incubate for 5–15 min at 37 °C; check after 5 min to see whether the cells detach from the dish.

5.4. Neutralize the reaction using 1 mL of trypsin inhibition solution and 2 mL of complete culture medium + chelexed-FBS by gentle pipetting. Next, transfer the cell suspension into the same 15 mL conical tube as in step 5.1.

5.5. Centrifuge the cell suspension at 100 x *g* for 5 min at 4 °C. Aspirate the supernatant with a pipette and resuspend the cell pellet in 1 mL of complete culture medium.

5.6. Count the cells using a hemocytometer. Next, plate 1 mL of the cell suspension into a new 24- or 6-well culture plate.

NOTE: Some of the cells from the early passages can be frozen in a mixture of 70% complete culture medium + 20% chelexed-FBS + 10% DMSO. ~1–2 cryovials of cells can be collected from one confluent culture plate.

6. Cryopreservation and recovery of keratinocytes

6.1. Cell freezing

6.1.1. Grow keratinocytes to 80%–90% confluency.

NOTE: Do not allow cells to overgrow, as this could reduce their proliferation status and viability.

6.1.2. Treat the keratinocytes in the dish with 0.05% trypsin-EDTA, as described in steps 5.1–5.5.

6.1.3. Count the keratinocytes using a hemocytometer. Prepare cryovials based on calculated cell numbers to allow for the transfer of 1 x 10^6 cells/mL to each vial.

6.1.4. Centrifuge the cell suspension at 100 x *g* for 5 min at 4 °C. Discard the supernatant and resuspend the cell pellet in a 10 mL solution of 10% DMSO + 20% chelexed-FBS + 70% complete culture medium (9 mL of complete medium + chelexed-FBS and 1 mL of DMSO).

6.1.5. Dispense the cells into cryovials at 1 mL of suspension per vial. Place the vials in a cryogenic storage container overnight at -80 °C. Transfer the vials to a liquid nitrogen tank the following day.

6.2. Cell recovery

6.2.1. Remove a cryovial from the liquid nitrogen tank and partially thaw at room temperature. In a 15 mL tube, mix 1 mL of the cell suspension with 3 mL of complete culture medium + chelexed-FBS.

6.2.2. Centrifuge the mixture for 5 min at 100 x *g* and 4 °C. Discard the supernatant and resuspend the pellet in 1 mL of complete culture medium + chelexed-FBS.

6.2.3. Plate the cell suspensions into new 6 cm Collagen I-coated culture dishes. Replace the culture medium every 2–3 days and passage the cells once confluent.

7. Immunofluorescent staining

7.1. Culture oral keratinocytes on square coverslips (22 mm x 22 mm) in 6-well plates (5 x 10^5 cells/well) for 2 days.

7.2. Fix keratinocytes in a solution of 4% paraformaldehyde (PFA) and PBS for 20 min at room temperature before washing three times with 1x PBS.

7.3. Permeabilize cells in a solution of 0.1% Triton in PBS. Incubate cells in blocking reagent (2.5% goat serum, 2.5% donkey serum) for 1 h at room temperature, followed by overnight incubation with primary antibodies (see **Table of Materials**) at 4 °C.

NOTE: Primary antibodies were used at the following dilutions: rabbit anti-K14 (1:1000), rat α6-integrin (1:100), rabbit anti-p63 (1:500), rabbit anti-K13 (1:100), and goat anti-PDGFRα (1:100).

7.4. Wash samples in a solution of 0.1% Triton in PBS, followed by incubation with secondary antibodies for 1 h at room temperature.

NOTE: The secondary antibodies (Alexa 488 or 555) were used at a 1:300 dilution.

7.5. Counterstain all samples with Hoechst solution for 10 min and mount cells onto glass slides.

NOTE: Hoechst solution is used to stain the nuclei of cells.

7.6. Perform sample imaging using a confocal microscope.

NOTE: The brightness and contrast are adjusted to equal intensity using image editing software.

REPRESENTATIVE RESULTS:

Overview of the dissection process and isolation of oral keratinocytes from the adult mouse palate

Dissociated oral keratinocytes were collected from the adult mouse palate and cultured in a customized 20% low-calcium chelexed-FBS formulation. The mouse palate consists of the hard palate and the soft palate (**Figure 1C**). The procedure for the isolation of mouse oral keratinocytes is summarized in **Figure 1D**. The palate tissue is dissected and transferred to a media containing an antibiotic-antimycotic solution before being incubated in 0.025% trypsin solution at 4 °C overnight. The following day, the palate tissue is treated with trypsin inhibitor solution and complete culture medium in equal volumes. Subsequently, the tissues are scraped using a surgical scalpel blade to collect oral keratinocytes. The cell suspension is filtered through a 100 µm cell strainer and centrifuged. The cells are then seeded in Collagen I-coated 24-well plates containing 2 mL of complete culture medium + chelexed-FBS.

Representative results of the successful isolation of mouse oral keratinocytes

Primary oral keratinocytes grew as a monolayer and displayed a cobblestone morphology (**Figure 2**). Small keratinocyte colonies were visible at 3–5 days (**Figure 2A,B**); these grew larger and formed tight colonies at 1 week of incubation (**Figure 2C**). Keratinocyte colonies displayed the typical morphological features of basal keratinocytes, indicating their healthy conditions. Human oral keratinocytes remained undifferentiated for several passages in the complete culture medium containing 0.06 mM Ca^{2+}[16,28]. The first passage was performed approximately 2 weeks from the initial plating (**Figure 2D**). At later passages, keratinocytes exhibited stable growth with a shorter period of culture (**Figure 2E–G**). Keratinocytes stopped growing if significant fibroblasts contamination occurred during the isolation process (**Figure 2H**).

Isolated mouse oral keratinocytes express basal keratinocyte markers

To confirm the status of primary oral keratinocytes, immunostaining was performed using the basal cell markers Keratin 14 (K14) and α6-integrin[34]. K14 and α6-integrin were expressed in keratinocytes after culturing (**Figure 3A,B**). The cells were also stained with stem cell marker p63 to confirm their stemness. Early passage (passage 4) and late passage (passage 7) cells showed uniform expression of p63 (**Figure 3C,D**). In contrast, keratinocytes treated with high calcium (1.2 mM induction for 2 days) exhibited decreased p63 expression (**Figure 3E**), indicating that high calcium treatment suppresses stem cell-related genes in primary keratinocytes as previously reported[16]. The differentiation marker Keratin 13 (K13) showed rare or no expression in both early and late passages and significant expression under high calcium treatment (**Figure 3F–H**). To test the possibility of fibroblast contamination in the keratinocyte culture, staining using the fibroblast marker PDGFRα was performed with the same set of keratinocytes compared with mouse embryonic fibroblast (MEFs). There was no expression of PDGFRα in the keratinocyte culture, compared with the high expression observed in MEF cells (**Figure 3I–3L**). These results indicated that this protocol could successfully isolate basal keratinocytes and maintain these cells in the undifferentiated state.

FIGURE LEGENDS:

Figure 1: Overview of the dissection procedure and isolation of mouse oral keratinocytes. (**A**) Schematic representation of the mouse oral cavity. (**B**) Instruments used to dissect the palate and isolate mouse oral keratinocytes. (**C**) Brightfield image of the mouse palate. Scale bar: 100 µm. (**D**) Summary of the protocol.

Figure 2: Representative results of the successful isolation of mouse oral keratinocytes. (**A–G**) Time-course images of cultured primary oral keratinocytes at 3 days (**A**), 5 days (**B**), 1 week (**C**), and 2 weeks (**D**) of culture after isolation. Morphologies of mouse oral keratinocytes after the first (**E**), second (**F**), and third (**G**) passages are shown. (**H**) Example of fibroblast contamination in mouse oral keratinocyte culture. Scale bar: 400 µm.

Figure 3: Isolated mouse oral keratinocytes express basal keratinocyte markers. (**A–B**) Representative images of immunofluorescent staining of K14 (**A**; red) and α6-integrin (**B**; green) in passage 4. (**C–E**) Representative images of immunofluorescent staining of p63 (green) in passage 4 (**C**), passage 7 (**D**), and high calcium treatment (**E**). (**F–H**) Immunostaining images of K13 (green) in passage 4 (**F**), passage 7 (**G**), and high calcium treatment (**H**). (**I–L**) Immunostaining images of PDGFRα (red) in passage 4 (**I**), passage 7 (**J**), high calcium treatment (**K**), and MEFs (**L**). Nuclei are stained with Hoechst (blue). Scale bars: 100 µm.

DISCUSSION:

Primary keratinocytes isolated from human or mouse skin epidermis have been utilized for many years in research and clinical applications[12,13,15,18,27–29]. By contrast, few protocols have been established to isolate and culture primary oral keratinocytes from adult mice[30–32]. The present study used a commercial complete culture medium and chelexed-FBS to maintain keratinocytes in a proliferative or stem cell-like state. This culture system can be employed in molecular and biochemical assays to further understand the features of oral epithelial stem cells and their related diseases.

Several critical steps are included in this protocol. Firstly, the trypsin concentration and the incubation time are essential in producing viable cells for subsequent cultures. We consistently used 0.025% trypsin solutions and 16 h incubation periods in the chamber hood at room temperature. If not incubated for a sufficient length of time, keratinocytes would not properly dissociate from the tissue, resulting in a lower final cell yield. Secondly, gentle pipetting of the cell suspension on the second day notably affects cell viability. Scraping should gently start from the epithelial side and not exceed 10 min per tissue sample. Finally, the first isolated cell suspension contains fibroblasts and other cell types; these unwanted cells will usually begin to disappear in subsequent cultures.

Potential limitations were identified during cell isolation and culture. In rare cases, fibroblasts may be contaminated during the isolation process, and the fibroblasts may inhibit the growth of keratinocytes in subsequent passages (**Figure 2H**). It is necessary to select a commercial medium that contains a fibroblast growth inhibitor to eliminate such contamination in the culture. Because the mouse palate and other oral mucosa have relatively small sizes, the initial cell yield from one

mouse may be low. Therefore, the entire culture period of this protocol—until the cryopreservation stage—is longer than that for primary skin keratinocytes. Isolated oral keratinocytes are best used within 10 passages, as more extended culture periods could change the cell properties and lower the number of stem cells.

The current method showed that mouse oral keratinocytes exhibited a tightly packed, cobblestone morphology and formed monolayer colonies under proliferative conditions. They also showed high expression of the basal markers α6-integrin, K14, and stem cell marker p63. In future studies, in addition to immunofluorescence staining, RNA-sequencing, RT-PCR, and western blot analyses will be used to verify the cellular heterogeneity and purity of oral keratinocytes, which will further enhance our understanding of the nature of these cells.

After 2–3 passages, oral keratinocytes were stable enough to be used in further functional experiments. Importantly, this culture protocol can be combined with transgenic mouse lines, including gene knockout, Cre-loxP, and tet-inducible systems, and can also be used in cellular and molecular assays. Thus, the present protocol provides researchers with a fundamental and efficient method that could be used to understand oral keratinocyte stem cell biology further.

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DISCLOSURES:

The authors declare no competing interests.

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Figure 1

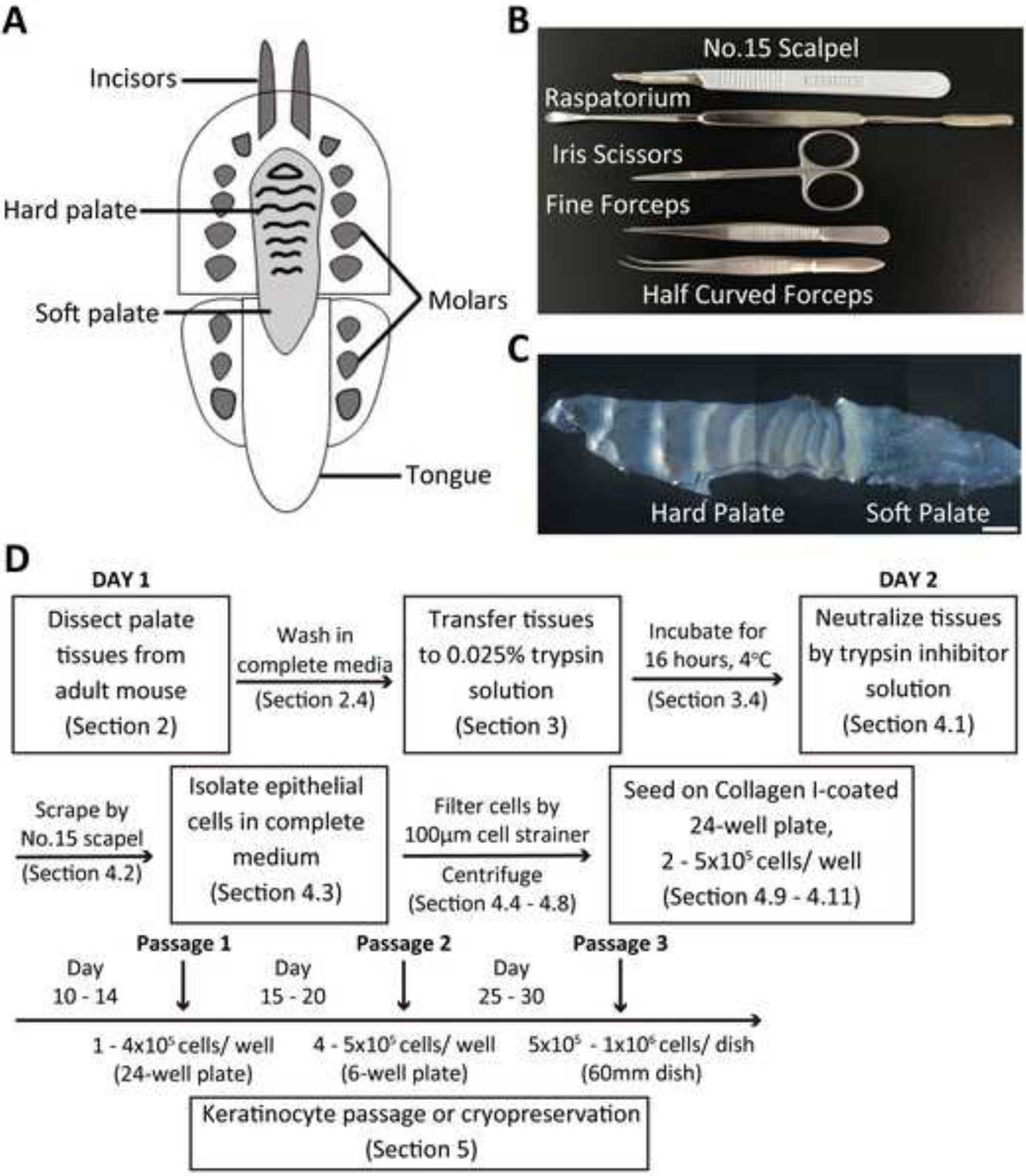

A
Incisors
Hard palate
Soft palate
Molars
Tongue
B
No.15 Scalpel
Raspatorium
Iris Scissors
Fine Forceps
Half Curved Forceps
C
Hard Palate
Soft Palate
D
DAY 1
Dissect palate tissues from adult mouse (Section 2)
Wash in complete media (Section 2.4)
Transfer tissues to 0.025% trypsin solution (Section 3)
Incubate for 16 hours, 4°C (Section 3.4)
DAY 2
Neutralize tissues by trypsin inhibitor solution (Section 4.1)
Scrape by No.15 scapel (Section 4.2)
Isolate epithelial cells in complete medium (Section 4.3)
Filter cells by 100µm cell strainer
Centrifuge
(Section 4.4 - 4.8)
Seed on Collagen I-coated 24-well plate, 2 - 5x10⁵ cells/ well (Section 4.9 - 4.11)
Passage 1
Passage 2
Passage 3
Day 10 - 14
Day 15 - 20
Day 25 - 30
1 - 4x10⁵ cells/ well (24-well plate)
4 - 5x10⁵ cells/ well (6-well plate)
5x10⁵ - 1x10⁶ cells/ dish (60mm dish)
Keratinocyte passage or cryopreservation (Section 5)

Figure 2

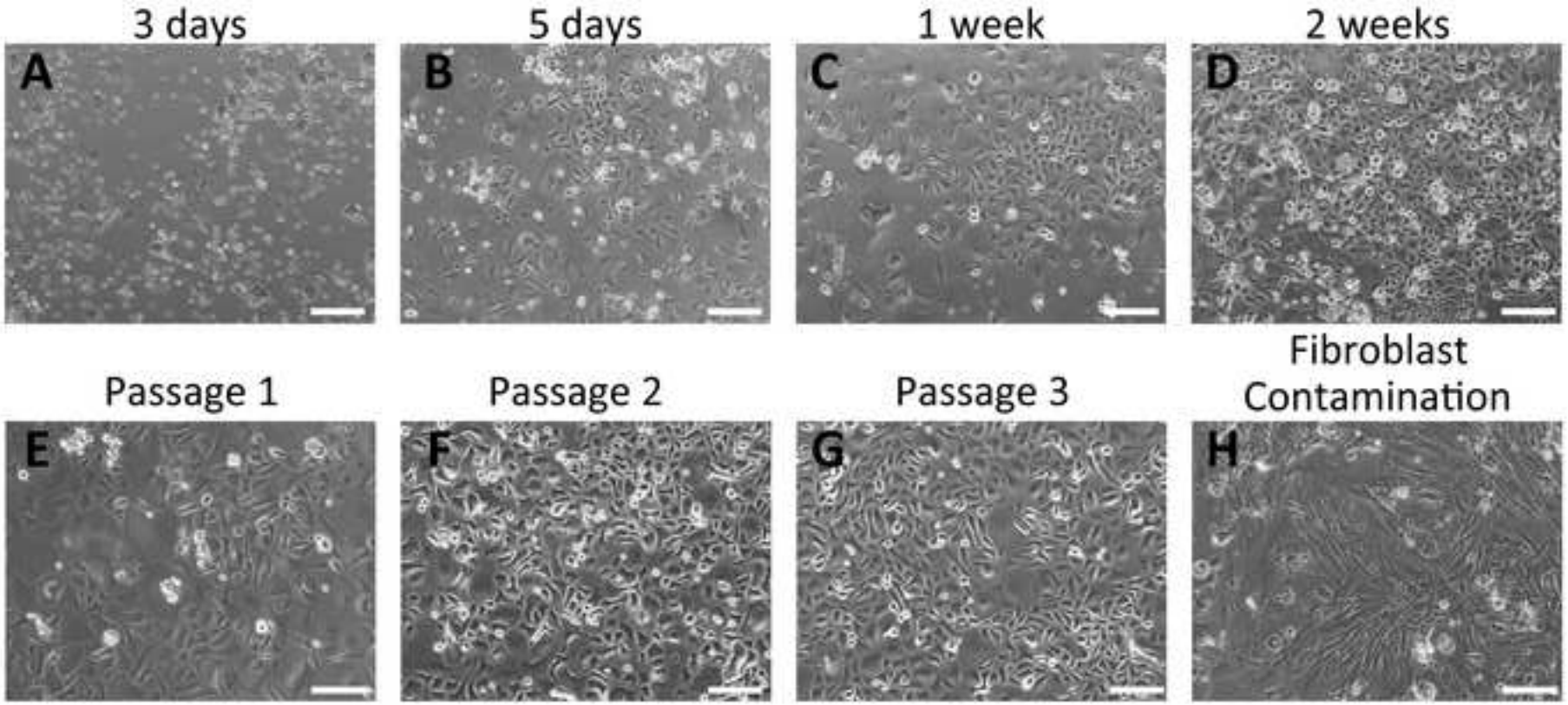
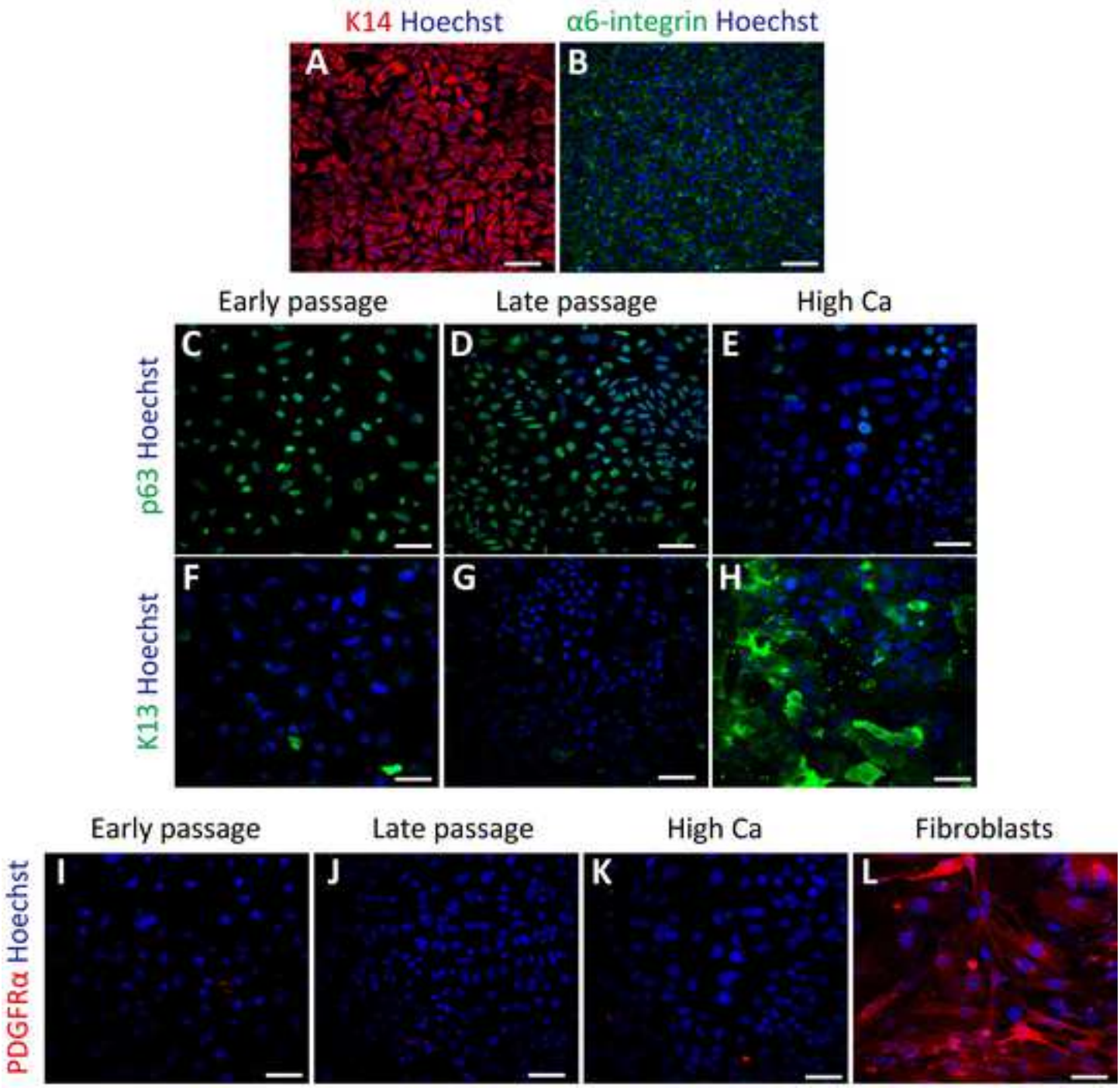


Figure 3



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.