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Isolation and Culture of Primary Human Gingival Epithelial Cells using Y-27632

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TITLE:

Isolation and Culture of Primary Human Gingival Epithelial Cells using Y-27632

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SUMMARY:

Here we present a modified method for the isolation and culture of human gingival epithelial cells by adding the Rock inhibitor, Y-27632, to the traditional method. This method is easier, less time-consuming, enhances stem cell properties, and produces larger numbers of high-potential epithelial cells both for the laboratory and for clinical applications.

ABSTRACT:

The gingival tissue is the first structure that protects periodontal tissues and plays meaningful roles in many oral functions. The gingival epithelium is an important structure of gingival tissue, especially in the repair and regeneration of periodontal tissue. Studying the functions of gingival epithelial cells has crucial scientific value, such as repairing oral defects and detecting the compatibility of biomaterials. As human gingival epithelial cells are highly differentiated keratinized cells, their lifespan is short, and they are difficult to passage. So far, there are only two ways to isolate and culture gingival epithelial cells, a direct explant method and an enzymatic method. However, the time required to obtain epithelial cells using the direct explant method is longer, and the cell survival rate of the enzymatic method is lower. Clinically, the acquisition of gingival tissue is limited, so a stable, efficient, and simple *in vitro* isolation and culture system is needed. We improved the traditional enzymatic method by adding Y-27632, a Rho-associated kinase (ROCK) inhibitor, which can selectively promote the growth of epithelial cells. Our modified enzymatic method simplifies the steps of the traditional enzymatic method and increases the efficiency of culturing epithelial cells, which has significant advantages over the direct explant method and the enzymatic method.

INTRODUCTION:

The human gingiva, the first line defense structure that protects periodontal tissue, is not only a physical and chemical barrier[1], but also secretes different classes of inflammatory mediators that participate in immune responses and constitute an immune barrier[2,3]. The gingival epithelium plays an important role in the repair and regeneration of periodontal tissue. Therefore, studying the defense and immunity of the gingival epithelium is of great significance for understanding the occurrence, diagnosis, and treatment of periodontitis. The isolation and culture of gingival epithelial cells from human gingival tissue is the first step required for studying the gingival epithelium. Such a procedure requires basic operations such as producing seed cells for tissue engineering, *in vitro* models of periodontal associated diseases, and materials for repairing periodontal defects.

Primary gingival epithelial cells are characterized by a low division rate *in vitro*[4]; researchers have been looking for an optimal isolation and cultivation method for decades. To date, two different techniques, a direct explant method and an enzymatic method, are commonly used in laboratories to obtain primary gingival epithelium cells *in vitro*[4,5]. The direct explant method has advantages such as the requirement of a lower amount of tissue specimens and simple isolation procedure, but it has the disadvantages of longer culture time and susceptibility to contamination[5]. Although the enzymatic method shortens the culture time required, the efficiency is relatively low and varies depending on the enzymes and medium used. Kedjarune

et al.[6] showed that the direct explant method, which requires more time before subculture (2 weeks), appeared to be more successful for culturing gingival epithelial cells compared to the enzymatic method. However, comparing these two methods, Klingbeil et al.[7] found that the enzymatic method had the best results for primary cultures of oral epithelial cells, and it was possible to obtain the optimal cell yield within the shortest time period (11.9 days versus 14.2 days).

Therefore, it was important to develop a more convenient and effective method for the isolation and culture of oral epithelial cells[4]. We previously reported that adding Y-27632, an inhibitor of Rho-associated protein kinase (ROCK), simplifies the isolation procedure of human primary epidermal cells and keratinocytes from adult skin tissues[8–10]. We developed G-medium, a new conditioned inoculation medium that spontaneously separates epidermal from dermal cells and supports the growth and yield of primary epidermal cells[8–10]. In the present study, we developed a new serum-free isolation and culture technique for gingival epithelial cells by combining G-medium with Y-27632. In essence, our method is based on a simplification of the traditional two-step enzymatic method, so we compared our new method with the direct explant method. This modified enzymatic method significantly shortens the time required to separate gingival epithelial cells from gingival tissue and increases the efficiency of culturing gingival epithelial cells.

PROTOCOL:

Human tissues used in this protocol are fresh adult gingival tissues discarded from extractions of impacted teeth in the Maxillofacial Surgery Department according to the guidelines of the Institution's Human Research Ethics Committee (Protocol No. GR201711, Date: 02-27-2017).

1. Preparations

1.1. Collect fresh adult gingival tissues in 15 mL tubes containing 10 mL of phosphate-buffered saline (PBS) supplemented with 3% penicillin/streptomycin (P/S) and keep the tissues at 4 °C.

NOTE: Treat the tissues as detailed below within 24 h after excision.

2. Prepare reagents and culture medium

2.1. Prepare the washing solution: 3% P/S. Mix 50 mL of PBS with 1.5 mL of penicillin (100 U/mL) and 1.5 mL of streptomycin (100 mg/L).

2.2. Prepare 500 mL of growth-factor-containing medium (called G-medium): DMEM/F12 (3:1) medium containing 1% P/S, 2% B27 supplement, 20 ng/mL of epidermal growth factor (EGF), 40 ng/mL of fibroblast growth factor 2 (FGF2), and 40 µg/mL of fungizone.

2.3. Prepare enzyme digestion solutions for the new method: Dissolve 125 mg of Dispase

powder and 125 mg of Type I collagenase powder in 50 mL of DMEM.

NOTE: Filter all the enzyme solutions through a 0.22 µm strainer and store at 4 °C.

2.4. Prepare 500 mL of the medium to neutralize the enzymatic digestion: 10% fetal bovine serum (FBS) and 1% P/S in the DMEM medium.

3. Direct explant method

3.1. Gingival tissue pretreatment

3.1.1. Wash gingival tissue with 5 mL of 75% ethanol for 30 s. Then, rinse twice with 5 mL of the washing solution (step 2.1) for 5 min.

NOTE: Perform all washes in cell culture dishes with a diameter of 50 mm.

3.2. Gingival epithelial cell culture

3.2.1. Cut the gingival tissue into 1 mm^3 pieces and scatter them in a 100 mm cell culture dish. Use fine pointed forceps and ophthalmic scissors for the cutting operations.

3.2.2. Add the G-medium (step 2.2) to the culture dish. Then, incubate the culture dish in a 5% CO_2 incubator at 37 °C. Use an inverted microscope (40x) to examine the tissues every 24 h.

3.2.3. Remove the gingival tissue pieces when the epithelial cells have propagated to a 2–5 mm in diameter around the tissue pieces. Change the G-medium every 2–4 days.

4. The new modified enzymatic method

4.1. Gingival tissue pretreatment

4.1.1. For this, follow the same steps as described for the direct explant method, step 3.1.1.

4.2. Digestion of gingival tissue

4.2.1. Cut the gingival tissue into tiny pieces, approximately <1 mm in size, with two sterilized blades. Repeat the process of shredding with two surgical blades.

4.2.2. Add 1 mL of 2.5 mg/mL dispase + collagenase solution to a 1.5 mL centrifuge tube

containing the gingival tissue pieces, and then incubate the tube for 15–20 min in an incubator at 37 °C.

4.2.3. When the tissue becomes transparent and flocculent, add 1 mL of the neutralization solution to terminate the digestion. Thoroughly mix the solution by pipetting up and down 10–15 times. Pass the solution through a 100 µm mesh filter.

4.2.4. Centrifuge at 200 x *g* for 5 min.

4.2.5. Remove the supernatant and resuspend the cell pellet at the bottom in 10 mL of growth-factor-containing medium (step 2.2). Transfer the cell suspension to a 100 mm cell culture dish. Add 10 µM of Y-27632 to the cell culture dish.

4.2.6. Culture the cells at 37 °C in a 5% CO_2 incubator. Replace the old G-medium with the fresh G-medium every 2 days. Observe the cells on Day 1 and Day 3.

5. Cell passaging

5.1. Take the dish out of the incubator, remove the spent medium and wash twice with PBS. Add 2 mL of 0.05% trypsin for each 100 mm dish.

NOTE: Shake the dish to make sure there is enough contact between the Trypsin solution and the dish bottom.

5.2. Leave the dish in an incubator around 5 min at 37 °C for the digestion process.

5.3. Use a microscope (40x) to examine the cells and make sure that most cells have separated from the bottom of the dish.

5.4. Add 2 mL of the neutralization solution to stop the digestion and transfer the cells into a 15 mL tube. Pipette up and down 10–15 times. Centrifuge the cells at 200 x *g* for 5 min.

5.5. Remove the supernatant slowly. Resuspend the cells with 10 mL of G-medium and count the number of cells.

5.6. Add about 1 x 10^6 cells in 10 mL of G-medium and 10 µM Y-27632 to each 100 mm dish.

5.7. Renew the G-medium and Y-27632 every 2 days. View the cells on Day 1 and Day 5.

REPRESENTATIVE RESULTS:

Figure 1 shows a schematic diagram of the direct explant method and the modified enzymatic

method. The direct explant method does not need any digestive enzyme during the whole process. In contrast, the traditional enzymatic method usually needs two sets of digestive enzymes, dispase and collagenase, to separate the epithelial sheet from the underlying fibroblast layer, and then trypsin to release the epithelial cells into suspension. Our new method omits the step of separation and is a simplified enzymatic method. Moreover, adding Y-27632 to the G-medium effectively promotes epithelial cell growth. The direct explant method usually takes about 2 weeks for the gingival epithelial cells to grow sufficiently to passage and is easily contaminated. However, the number of gingival epithelial cells obtained by the new method is significantly larger than that obtained by the direct explant method and takes only an average of 8 days to meet the requirements for passaging.

[place Figure 1 here]

Adult gingival epithelial cells prepared by the direct explant method and by the new method were observed in a microscope on the third and seventh days (**Figure 2A**). It can be seen that the new method significantly increased the production of gingival epithelial cells on the third and seventh days. The growth curves of gingival epithelial cells obtained by the new method and by the direct explant method were measured using a cell counting kit (CCK-8) at different time points (1d, 2d, 3d, 4d, 5d, 6d) (**Figure 2B**). The doubling time of cells prepared using the new method was about 1–2 days, but the doubling time of cells prepared using the direct explant method was about 5–6 days. The cell yields by the two methods were calculated on the seventh day (**Figure 2C**) and showed that the number of cells produced by the new method (9×10^6) was more than three times higher than the number of cells produced by the direct explant method (3×10^6). **Figure 2D** shows that it took about half the time for the new method (6 days) to achieve 80% confluence compared with the direct explant method (13 days).

[place Figure 2 here]

Immunofluorescence analysis of gingival epithelial cells (at passage 3) obtained by the new method showed that the expression of CK5, CK18, and Pan-CK (CK14, CK15, CK16, and CK19) was positive (**Figure 3A,C,D**)[11-15], while the expression of CK10 and vimentin was low (**Figure 3B,E**)[11,16]. CK5 and Pan-CK (CK14, CK15, CK16, and CK19) were mainly localized in the basal layer of epithelial cells, which is a sign of their high differentiation potential[11,14,15]. CK10 is a marker of differentiated epithelium[11], and vimentin is a marker of gingival fibroblasts[16]. The keratin positive rate of gingival epithelial cells isolated by the new method was higher, especially CK5, CK18, and Pan-CK (**Figure 3A,C,D**), which indicated that the isolated gingival epithelial cells could maintain a good basement membrane function. The low expression of the differentiation marker CK10 (**Figure 3B**) indicated that the gingival epithelial cells had a high differentiation potential. *In vitro* cultures showed that the gingival epithelial cells isolated

by the new method could be cultured to the eighth generation, and the low expression of vimentin indicated that the gingival epithelial cells had a high purity, that no gingival fibroblasts contaminated them and that the isolation efficiency was high (**Figure 3E**).

[place Figure 3 here]

The new method increased the expression of ki67, p63, and p75NGFR (low affinity nerve growth factor receptor p75) in primary gingival epithelial cells. Passage 3 cells were selected for Immunofluorescence analysis, which revealed that the positive expression of ki67, p63, and p75NGFR was higher than that of gingival epithelial cells obtained by the direct explant method (**Figure 4A,C,E**). The positive expression rates of ki67, p63, and p75NGFR were 80%, 98%, and 96% in the new method, respectively, which were significantly higher than those of ki67 (35%), p63 (40%), and p75NGFR (47%) obtained by the direct method (**Figure 4B,D,F**). Ki67, p63, and p75NGFR are markers of oral epithelial stem cells[17]. These results indicated that the primary gingival epithelial cells isolated and cultured using the new method contained stem cell populations, had a high proliferation potential, and maintained the stem cell properties of gingival epithelial cells.

[place Figure 4 here]

FIGURE LEGENDS:

Figure 1: Comparison of the direct explant method and the new method. (**A**) Scheme showing the process of the direct explant method. The gingival tissue pieces are placed in a culture dish. The epithelial cells are cultured in G-medium and grow out from the tissue pieces. The direct explant method usually takes about 13 days for the epithelial cells to reach 80% confluence. (**B**) Scheme showing the process of the new enzymatic method. The gingival tissue fragments are digested by dispase and collagenase I, after which the cell pellets are cultured in G-medium with Y-27632. The new enzymatic method usually takes about 6 days to reach 80% confluence which is suitable for passaging.

Figure 2: The new enzymatic method increases the production of primary gingival epithelial cells. (**A**) Images of gingival epithelial cells prepared by the direct explant method (bottom row) and the new enzymatic method (top row) on the third and seventh day after initial inoculation. Scale bars = 200 μm. (**B**) Gingival epithelial cells cultured by the direct method and the new enzymatic method were collected on days 1, 2, 3, 4, 5, and 6, and the number of gingival epithelial cells was analyzed using a CCK-8 kit. (**C**) After 7 days of culture, gingival epithelial cells were detached by trypsin and were collected. A cell counting plate was used to determine the number of gingival epithelial cells prepared separately by the two methods. The number of cells was calculated from the two methods, which were repeated three times separately, and the results are expressed as the average number of cells per 100 mm dish. (**D**) The average time to reach 80% confluence of primary gingival epithelial cells (passage 0)

prepared by the new enzymatic method and by the direct explant method is compared. B, C, and D: Student's *t*-test was used; the error bars show the standard deviation; n = 4; **p < 0.01, *p < 0.05 when comparing the new enzymatic method with the direct explant method.

Figure 3: Specific markers of gingival epithelial cells (P3) prepared by the new enzymatic method. (**A–E**) show CK5 (red), CK10 (red), CK18 (red), Pan-CK (red), and vimentin (red) positive cells, DAPI (blue) was used to stain nuclei. (**A–C**) scale bars = 100 µm; (**D**) and (**E**) scale bars = 50 µm; the experiment was repeated four times independently. (**A**), (**C**), and (**D**) show the positive expression of CK5, CK18, and pan-ck (CK14, 15, 16, and 19) in gingival epithelial cells, while (**B**) and (**E**) show the low expression of CK10 and vimentin in gingival epithelial cells.

Figure 4: Stem cell characteristics of gingival epithelial cells (P3) obtained by the direct explant method and the new enzymatic method. (**A**), (**C**), and (**E**) show representative immunofluorescence images of gingival epithelial cells at passage 3 (P3), respectively, showing ki67 (red), p63 (red), and p75NGFR (red) positive cells obtained by the direct explant method and the new enzymatic method. DAPI (blue) was used to stain nuclei. Scale bars = 50 µm. (**B**), (**D**), and (**F**) show quantitative analysis of ki67-positive cells, p63-positive cells, and p75NGFR-positive cells, respectively, in gingival epithelial cells (P3) obtained by the direct explant method and by the new enzymatic method. A total of 400 cells were counted to quantify each group, and the average numbers of ki67, p63, and p75NGFR positive cells are shown. B, D, and F: Student's *t*-test was used; the error bars show the standard deviation; the experiment was repeated four times independently (n = 4); **p < 0.01, when comparing the new enzymatic method with the direct explant method.

DISCUSSION:

The gingival tissue is a key structure that maintains periodontal integrity and health. Gingival epithelial cells have significant roles in the repair and regeneration of periodontal tissue and can be used in scientific research and clinical applications and related fields, including oral biology, pharmacology, toxicology, and oral mucosa deficiencies[18]. Therefore, it is necessary to develop a stable and efficient method to harvest oral epithelial cells[19]. Primary epithelial cells are a kind of fully differentiated cells with few passages and a short lifespan. Culturing epithelial cells has proven to be more complex than culturing fibroblasts[5].

At present, the published literature shows that various protocols exist for the isolation and culture of epithelial cells. However, two methods, the direct explant method and the enzymatic method, are the most frequently used among those protocols. In 1910, Carrel and Burrows first described a method to obtain gingival and buccal epithelial cells called the direct

explant method[20]. The direct explant method has the advantages of low-weight requirements for tissue specimens, less complicated procedures, less variation, and less involvement in the steps, but it has the disadvantages of requiring a long culture time and is highly susceptible to contamination[5]. In 1975, Rheinwald and Green first reported the enzymatic method using irradiated 3T3 mouse fibroblasts as a feeder layer to culture oral epithelial cells *in vitro*[21]. Although that method greatly improved the yield of keratinocytes, the irradiated mouse feeder cell layer had potential biological risks[22]. After that, culture systems without feeder-cells[22] and without serum[23,24] were developed which proved that 3T3 cells were not necessary for epithelial cell cultures. Although the enzymatic method requires less culture time, the efficiency is relatively low and varies depending on the enzymes and medium used[25]. Several laboratories compared the two methods and Kedjarune et al.[6] observed the direct explant technique to be more successful than the enzymatic method and found a higher rate of cell proliferation. Shwetha et al.[26] concluded that the direct explant method appears to be a simple and successful technique for the isolation of oral mucosal keratinocytes. However, Klingbeil et al.[7] concluded just the opposite, i.e., the enzymatic method showed the best results in less time with a good life span. A survey conducted in the UK by Daniels et al.[19] reviewed the isolation and cell culture methods of human keratinocytes and reported that 21 out of 34 laboratories that responded used the enzymatic method with some variations. Although the direct explant method requires less tissue and has fewer handling steps than the enzymatic method, it has been suggested that the enzymatic method is faster and easier to manage[6]. Therefore, it was necessary to develop a more convenient and effective method for the isolation and culture of oral epithelial cells[4]. Our new method, a simplified enzymatic method that uses Y-27632 significantly increased the production of gingival epithelial cells and shortened the time required to separate gingival epithelial cells from gingival tissue.

Figure 2A shows that the new method significantly increased the production of gingival epithelial cells on the third and seventh days. **Figure 2B** shows that the doubling time of cells prepared using the new enzymatic method was about 1–2 days but using the direct explant method took about 5–6 days. The number of cells produced using the new enzymatic method (9×10^6) was more than three times greater than the direct explant method (3×10^6) on the seventh day (**Figure 2C**). Cells prepared using the new enzymatic method took 13 days to become 80% confluent, which is consistent with previous studies, while the direct explant method took about 2 weeks[6], 20.25 ± 1.05 days[18] and 14.2 ± 2.76 days[7] for the cells to become fully confluent before subculture. However, the new enzymatic method took only 6 days to become 80% confluent, which is much shorter than the direct explant method of 13 days in our laboratory and Klingbeil et al.'s[7] enzymatic method of 11.9 ± 2.36 days. We also found an interesting phenomenon, that is, the epithelial cell colonies grew in a multi-layered structure in the direct explant method, while a uniform monolayer structure was observed using the new enzymatic method. Obviously, thicker multi-layered colonies prolong the time required to become 80%–100% confluent. The reason why the new enzymatic method promotes cell

proliferation is the addition of Y-27632, an inhibitor of ROCK1 and ROCK2. Y-27632 was initially reported for its positive effects on human epithelial cell proliferation and differentiation in 2008[27]. Chapman et al.[28] reported that treatment with Y-27632 greatly increased the proliferative capacity of keratinocytes and resulted in efficient immortalization without detectable cell crisis. In our previous studies, we discovered that Y-27632 simplifies the isolation procedure of human primary epidermal cells and keratinocytes from adult skin tissue[8–10]. Strudwick et al.[29] reported that the use of 10 μM Y-27632 together with low calcium allowed primary human keratinocytes to be cultured without a cell feeder layer for longer periods of time while retaining the ability to differentiate and form a stratified epithelium. In this study, the new enzymatic method using Y-27632 greatly increased the harvesting of epithelial cells by extending their lifespan and promoting their proliferative capacity.

The traditional enzymatic method contains two digestion operations. The first digestion is to separate epithelial tissue from connective tissue using dispase, which works from the stromal side[4,5]. The second digestion usually uses trypsin to separate epithelial cells from epithelial tissue[4,5]. Due to the destruction of epithelial cells by trypsin, the efficiency of the enzymatic method is affected[4]. The new enzymatic method described here is a simplified method that omits one digestion step. The main differences between the new and traditional enzymatic methods refer to the protocols of separating epithelial tissue from connective tissue[5]. Additionally, the new method uses dispase and collagenase instead of trypsin in the digestion step, and hence the integrity of the epithelial cells is well protected. Clinically, it is much more difficult to obtain the same amount of tissue from human gingiva than from the skin and buccal mucosa. The direct explant method required only small pieces of gingival tissue and cultivated a larger number of cells when compared with the enzymatic method[18]. We treated a small amount of gingival epithelial tissue using the new method and harvested a very satisfactory cell density. This shows that the new method does not depend on relatively large amounts of tissue to be initially provided. One possible limitation of the new method is that there could appear a small amount (<5%) contamination of fibroblasts at initial culture (passage 0), but it can be easily removed by a short-term treatment of 0.05% trypsin as reported previsouly[9].

In this study, CK5, CK18, and Pan-CK (CK14, CK15, CK16, and CK19) were positive (**Figure 3A,C,D**). This suggested that the isolated gingival epithelial cells were able to maintain their basement membrane function, which is consistent with the studies of Orazizadeh et al.[30] and Kedjarune et al.[6]. CK5 and CK14 are expressed by undifferentiated cells localized in the epithelial-basal layer. CK19 is typically found in simple epithelium and in non-keratinized stratified epithelium[31]. Immunofluorescence analysis of gingival epithelial cells (P3) obtained using the new enzymatic method showed that the expression levels of ki67, p63, and p75NGFR were higher than in cells obtained using the direct explant method (**Figure 4**). This result indicates that the new method can maintain the stem cell properties of gingival epithelial cells and promotes the proliferation potential of gingival epithelial cells. Our previous study found that Y-27632 maintains the skin epidermal stem cell potential after

expansion[8]. Wang et al. found that Y-27632 facilitates the proliferation, migration, and pluripotency of human periodontal ligament stem cells[32].

In summary, the new modified enzymatic method provides a simplified and effective procedure to isolate and culture human primary epithelial cells from adult gingival tissue. This new method is easier, less time-consuming and enhances their stem cell properties, and thus has obvious advantages compared to the direct explant method in many parameters. This advanced method is more suitable for producing large numbers of high-potential epithelial cells both for laboratory and for clinical applications.

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DISCLOSURES:

All the authors declare no conflicts of interest.

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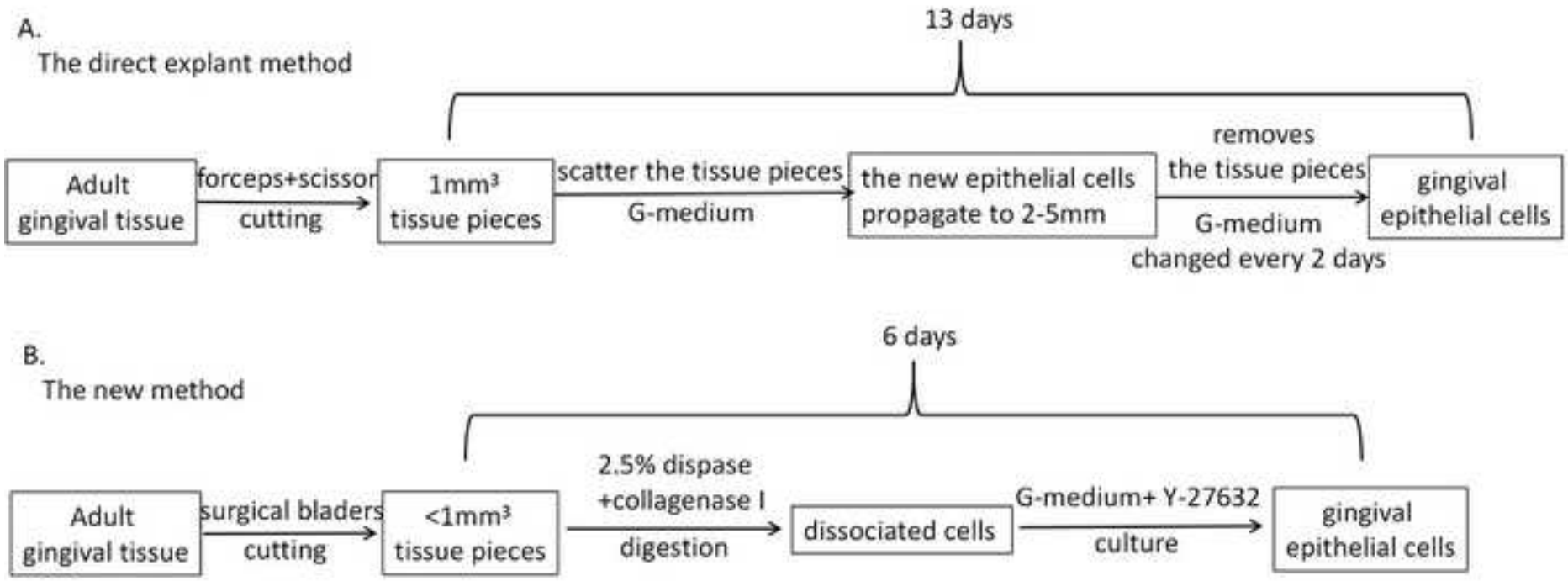
519

Figure 1



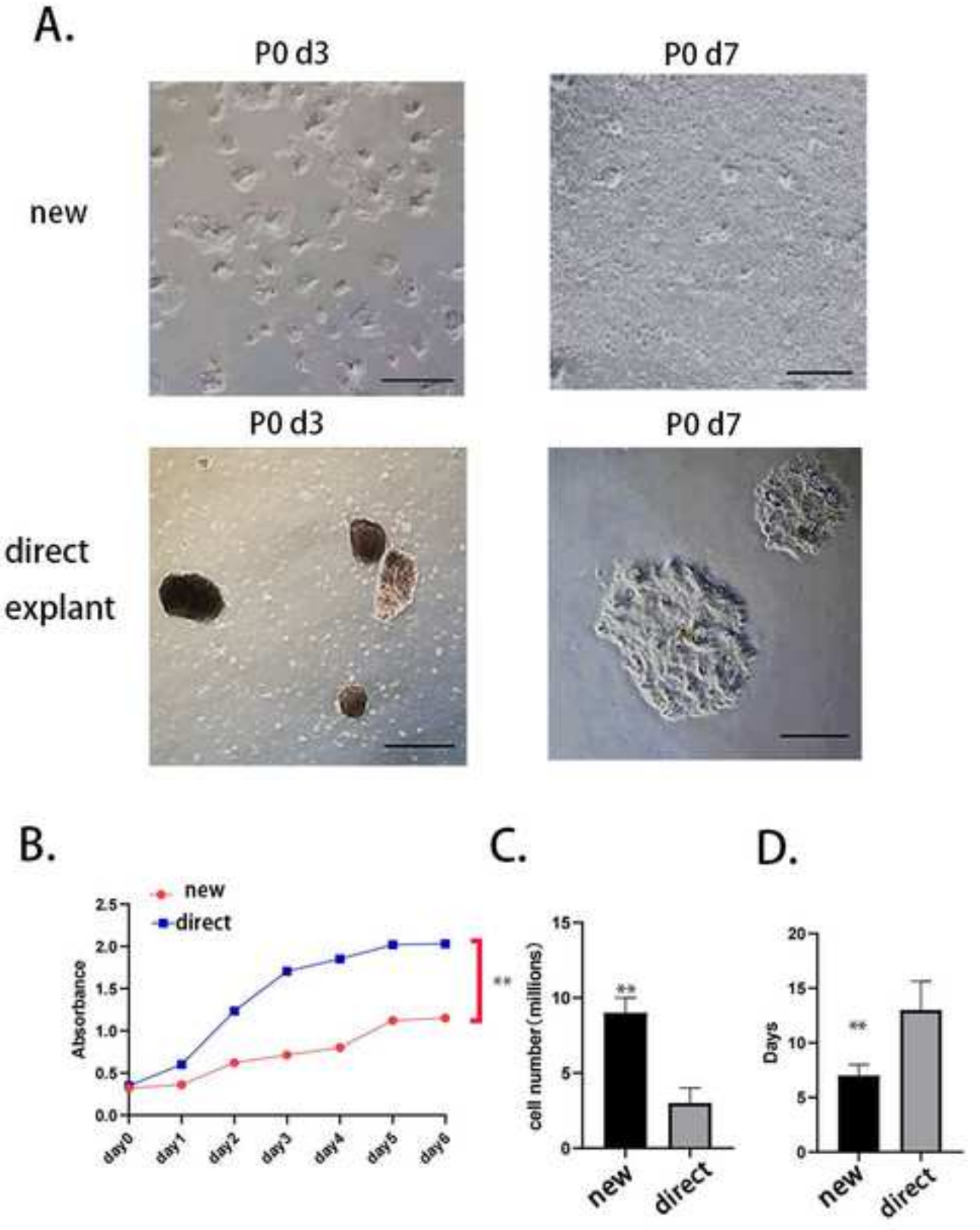


Figure 3

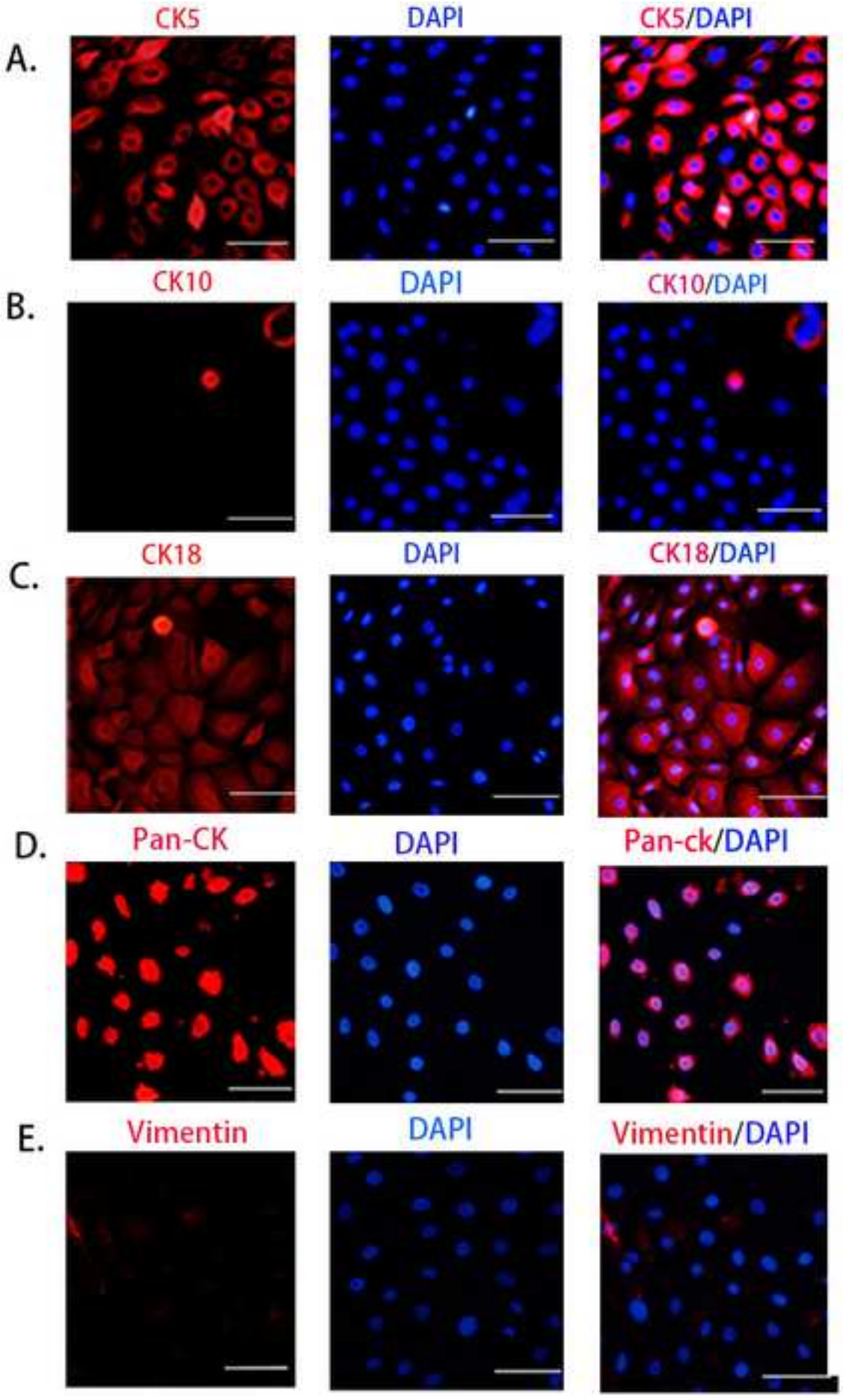
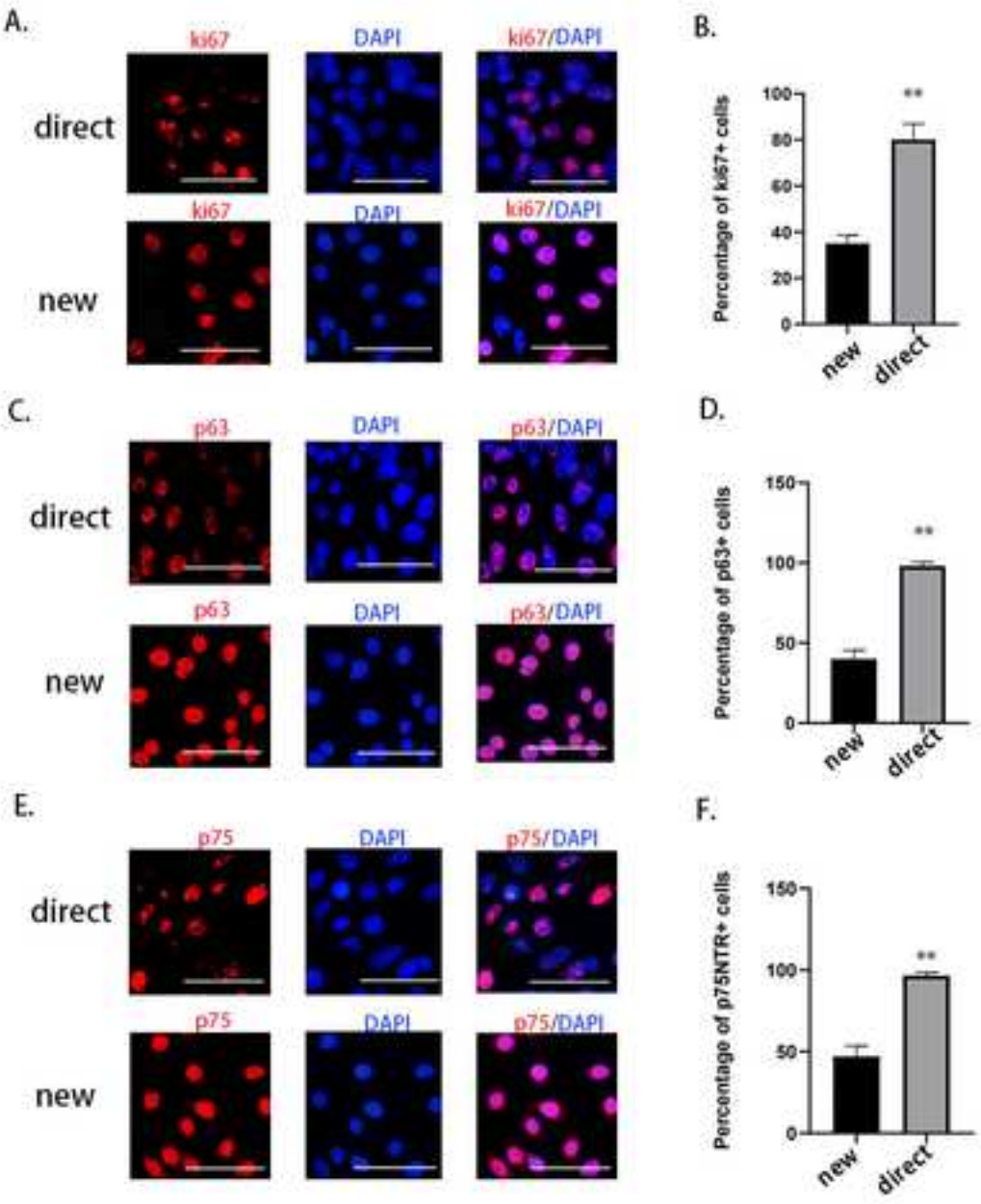


Figure 4



October, 10, 2021

Re: JoVE62978R2

Dear Dr. Vineeta Bajaj,

Appended is a revised version of our manuscript titled “Isolation and Culture of Primary Human Gingival Epithelial Cells using Y-27632”, which containing our responses to editorial comments and suggestions, together with the correspondingly revised video.

On behalf of our co-authors, we thank you very much for giving us an opportunity to revise our manuscript. We hope that you will find the revised version now suitable for publication in ***JoVE.*** We deeply appreciate your consideration, and we are looking forward to your positive response.

Sincerely,

Xunwei Wu, M.D, Ph.D

Video Produced by Author:more than 50 MB.

This piece of the submission is being sent via mail.