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Y-27632 Enriches the Yield of Human Melanocytes from Adult Skin Tissues

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TITLE:

Y-27632 Enriches the Yield of Human Melanocytes from Adult Skin Tissues

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KEYWORDS:

Y-27632, melanocytes, primary skin cell isolation, Rho-associated kinase inhibitor, skin pigmentation, adult tissues

SUMMARY:

This paper reports that the addition of Y-27632 to TIVA medium can significantly increase the yield of melanocytes from adult skin tissues.

ABSTRACT:

The isolation and culture of primary melanocytes from skin tissues is very important for biological research and has been widely used for clinical applications. Isolating primary melanocytes from skin tissues by the conventional method usually takes about 3 to 4 weeks to passage sufficiently. More importantly, the tissues used are usually newborn foreskins and it is still a challenge to

efficiently isolate primary melanocytes from adult tissues. We recently developed a new isolation method for melanocytes that adds Y-27632, a Rho kinase inhibitor, to the initial culture medium for 48 h. Compared with the conventional protocol, this new method dramatically increases the yield of melanocytes and shortens the time required to isolate melanocytes from foreskin tissues. We now describe this new method in more detail using adult epidermis to efficiently culture primary melanocytes. Importantly, we show that melanocytes obtained from adult tissues prepared by this new method can function normally. This new protocol will significantly benefit studies of pigmentation defects and melanomas using primary melanocytes prepared from easily accessed adult skin tissues.

INTRODUCTION:

The goal of this study was to develop a simple and effective protocol to isolate melanocytes from the epidermis of adult skin for biological research and clinical applications. Melanocytes, which are located in the basal epidermis of the skin and in hair follicles, play an important role in pigmentation of the skin and hair by producing melanin[1]. The resulting skin pigmentation from epidermal melanocytes acts as an ultraviolet radiation filter that reduces/prevents DNA damage to underlying cells in the skin[2]. The abnormal proliferation of melanocytes in the skin is quite common, such as in the formation of benign nevi (moles) in which melanocytes potentially transform to oncogenic growth followed by cellular senescence[3].

Since 1957, the isolation and subsequent culture of human primary melanocytes has been possible[4], but only since 1982 has there been an efficient method that can reproducibly establish cultures of human melanocytes from the epidermis[5]. The conventional method to isolate primary melanocytes from the epidermis involves a two-step enzymatic digestion. Briefly, the skin is initially digested with dispase to separate the epidermis from the dermis, after which the epidermis is digested with trypsin to produce suspensions containing melanocytes and keratinocytes, which can then be selectively grown in different media. Currently, the initial culture usually takes about 3 to 4 weeks for melanocytes to reach confluency using the conventional method, which is likely due to the low efficiency of their isolation. Therefore, increasing the initial production of melanocytes from adult skin tissues would be quite helpful for both laboratory research and clinical applications.

Many growth factors, most of which have been shown to be secreted by keratinocytes (e.g., α-MSH, ACTH, bFGF, NGF, ET-1, GM-CSF, HGF, LIF and SCF)[5-8], regulate the differentiation and proliferation of mammalian melanocytes. In the absence of feeder layers, the lack of those growth factors leads to the decreased proliferation of melanocytes and their increased apoptosis[9]. The co-culture of keratinocytes as feeder cells and melanocytes could lead to accelerated melanocyte proliferation and reduced apoptosis. However, the co-culture method not only demands more skin tissues to prepare keratinocytes, which is not practical, but also could not work efficiently because the culture conditions required by melanocytes does not favor keratinocyte growth, and vice-versa.

Previous studies have reported that the addition of Y-27632, a ROCK inhibitor, into the growth medium can enhance the yield of human primary epidermal cells from skin tissues[10-14]. Therefore,

it would be interesting to test whether the isolation of human primary melanocytes would benefit from the presence of Y-27632. Indeed, the addition of Y-27632 into the inoculation TIVA medium[15], which contains TPA, IBMX, Na_3VO_4 and dbcAMP, significantly increased the yield of primary melanocytes isolated from foreskin tissues in the initial cultures[16]. Compared to the conventional protocol, this new protocol is significantly more efficient in increasing the yield of melanocytes[16]. Therefore, this protocol describes the new method in detail using adult skin tissues based on a previous study[16] in order to promote its application in basic and applied biological research.

PROTOCOL:

The use of adult foreskin tissues in this protocol has been approved by the Human Research Ethics Committee (No.2015120401, date: May 12, 2015).

NOTE: Perform all the following procedures in a sterile environment to prevent contaminations of cells and cultures.

1. Preparations

1.1. Gather fresh adult foreskin tissues from circumcision surgeries in 15 mL tubes containing 10 mL of phosphate buffer saline (PBS) and store in 4 °C.

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

1.2. Prepare reagents and culture medium.

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

1.2.2. Prepare the termination solution (neutralization solution) for neutralization of enzymatic digestion. Supplement 1% P/S, 10% fetal bovine serum (FBS) into DMEM medium.

1.2.3. Prepare TIVA medium to culture melanocytes: Ham's F12; 10% FBS; 1x P/S/glutamine; 50 ng/mL 12-O-tetradecanoyl phorbol-13-acetate (TPA); 1 x 10^{-4} M 3-isobutyl-1-methyl xanthine (IBMX); 1 µM Na_3VO_4; 1 x 10^{-3} M N-6,2′-O-dibutyryladenosine-3′,5′-cyclic monophosphate (dbcAMP).

2. The conventional method

2.1. Skin tissue pretreatment

2.1.1. Rinse each adult foreskin tissue once with 10 mL of 75% ethanol (alcohol) for 30 s, and then rinse twice with 10 mL of washing solution (3% P/S), for 5 min each.

NOTE: Wash foreskin tissues with 10 mL of PBS in the beginning if there is a lot of blood. All washes are prepared in 100 mm cell culture dishes.

2.1.2. Scrape each foreskin tissue with a surgical blade to remove subcutaneous adipose tissues and loose connective tissues in the cover of a 100 mm cell culture dish.

NOTE: Use a scalpel to scrape the fat layers away until only the thin epidermis and the dense dermis remain.

2.1.3. Transfer each adult foreskin into another 100 mm culture dish with the dermis side down.

NOTE: Cut each adult foreskin tissue into 3-4 mm wide strips using a scalpel blade to make the dispase work more efficiently.

2.1.4. Add 2.5 mg/mL dispase to each 100 mm culture dish with adult foreskin tissues and incubate for 16 to 20 h at 4 °C.

NOTE: Each gram of tissue is digested with 10 mL of dispase solution.

2.2. Separation of the epidermis from the adult foreskin tissue

2.2.1. After digestion for 16 to 20 h, separate the epidermis from the dermis using tweezers. Grab one side of the dermal edge of the tissue with one tweezer and the corresponding position of the epidermal part with another tweezer and gently peel off the epidermis.

2.2.2. Wash the epidermis 3x with washing solution (3% P/S), and then transfer the epidermis into a tube.

2.2.3. Submerge the epidermis by adding 5 mL of 0.05% trypsin into the tube and incubate in a water bath for 30 min at 37 °C.

NOTE: Shake the tube to ensure the epidermis is completely submerged before incubation.

2.3. Collection and culture of primary cells

2.3.1. Add an equal volume of termination solution (10% FBS, 1% P/S in DMEM) to neutralize the trypsin and pipette the solution up and down 10-15 times to separate the epidermal cells.

2.3.2. Pass the cell suspension into a new 50 mL tube through a 100 µm mesh filter to remove debris.

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

2.3.4. Remove the supernatant and add 10 mL of inoculation TIVA medium to resuspend the

cells.

2.3.5. Seed the resuspended cells into a 100 mm culture dish.

2.3.6. Culture in a 37° C incubator with 5% CO_2.

2.3.7. Change the medium every 2 days.

2.3.8. Passage cells when they reach 80% confluence.

3. The new method

NOTE: The procedure for tissue preparation and digestion in the new method is the same as described above for the conventional method, the only difference being that the isolated fresh cells are resuspended in 10 mL of TIVA medium containing 10 µM Y-27632.

3.1. Two days after seeding, replace the media with normal TIVA medium without Y-27632. After that, the culture conditions are the same between the new and the conventional methods.

4. Cell passaging

4.1. Remove the TIVA medium and rinse each culture dish twice with PBS.

4.2. Add 2 mL of 0.05% trypsin per 100 mm cell culture dish.

NOTE: Shake the dish to ensure adequate contact between digestive enzymes and the bottom of the dish.

4.3. Digest in a 37 °C incubator for 2 min.

4.4. Use a microscope to check the cells, and make sure that most cells have dissociated from the cell culture dish.

NOTE: Gently tap the dish to release the cells to round up and float in the solution. Prolong the digestion time if most cells did not suspend after tapping, but no more than 5 more min.

4.5. Transfer the melanocytes into a 15 mL tube after neutralizing the trypsin activity by adding 2 mL of termination solution.

Centrifuge at 200 x *g* for 5 min.

4.6. Resuspend each cell pellet with 10 mL of TIVA medium after slowly removing the supernatant, and then count the number of melanocytes.

4.7. Plate about 1 x 10^6 melanocytes in 10 mL of TIVA medium per 100 mm cell culture dish.

4.8. Renew the cell culture medium every 48 h.

REPRESENTATIVE RESULTS:

Figure 1 shows a schematic diagram comparing the conventional and the new methods. The procedure for tissue preparation and digestion with the new method is the same as the procedure for the conventional method, the only difference being that the isolated cells are resuspended in 10 mL of TIVA medium in the presence of 10 μM Y-27632. Two days after seeding, replace the media with normal TIVA medium without Y-27632. The conventional method of isolating primary melanocytes from skin tissues usually requires about 3 to 4 weeks for melanocytes to grow in sufficient numbers to passage, but more importantly, the tissues usually come from newborn babies and it is very difficult to isolate primary melanocytes from adult skin tissues. However, using the new method, melanocytes from adult tissues are observed in significantly greater numbers than melanocytes isolated using the conventional method. Microscopic views were taken at days 3, 6 and 8 when isolating the melanocytes from adult skin tissue using the conventional method and the new method (**Figure 2A**). In these microscopic views, the new method significantly increased the yield of melanocytes at days 6 and 8. Melanocyte numbers were then quantified by counting at least 10 different microscopic fields at days 3, 6 and 8. The results showed that the number of melanocytes isolated using the new method is much higher than the number of melanocytes isolated using the conventional method at days 6 and 8 (**Figure 2B**). After 8 days of culture, the melanocytes were harvested using trypsin and then counted with an automated cell counter in each single 100 mm dish, which revealed that the new method increased the melanocyte yield by about five times (**Figure 2C**). Microscopic views taken at day 9, one day after passaging, showed that the passaged melanocytes proliferated normally (**Figure 2D**), which was confirmed by Ki67 staining (**Figure 2E**).

The enhanced yield of melanocytes by Y-27632 during the initial culture after isolation has been shown through the regulation of keratinocytes in a previous publication[16]. As shown in **Figure 3**, Y-27632 did not increase the growth and proliferation of passaged melanocytes (**Figure 3A-B**) and did not inhibit the apoptosis of melanocytes (**Figure 3C-D**). However, Y-27632 significantly increased keratinocyte attachment (**Figure 3E-H**) and also promoted its surviving (**Figure 3I**) in the melanocyte-culture TIVA media. Either co-culture with keratinocytes in the presence of Y-27632 or the conditioned medium derived from Y-27632 treated keratinocytes could significantly enhance the growth of melanocytes (**Figure 3J**).

To further characterize the melanocytes isolated using the new method, MITF, a specific melanocyte marker, was analyzed using immunofluorescence (IF) staining to check the purity of melanocytes after initial passaging. **Figure 4A** shows that the percentage of cells expressing MITF at passage 1 was nearly 100%, indicating that pure melanocytes were obtained after the first passage by the new method, which is similar to the conventional method. To test whether melanocytes isolated using the new method can function normally, they were treated with forskolin (FSK) for 2 days in vitro, which induced the levels of pigmentation of melanocytes (**Figure 4B**). Taken together, these data suggest that the melanocytes isolated using the new

method can function normally and can produce pigment.

FIGURE AND TABLE LEGENDS:

Figure 1: Comparison of the new method and the conventional method. This scheme shows a comparison of the conventional method with the new isolation method. The only difference is that the isolated fresh cells are resuspended in 10 mL of TIVA medium in the presence of 10 μM Y-27632. Two days after seeding, the medium is replaced with normal TIVA medium without Y-27632. The new method usually achieves a density of melanocytes suitable for passaging at around day 8, while the conventional method usually takes about 20 days to grow sufficient numbers of melanocytes for passaging.

Figure 2: The new isolation method increases the production of primary melanocytes. (**A**) Representative images of melanocytes prepared by the conventional method (top row) and by the new method (bottom row) at days 3, 6 and 8 after the initial inoculation. (**B**) Quantification of melanocyte numbers prepared using the conventional method and the new method by counting melanocyte numbers in at least 10 different microscopic fields (cell number/vision) at days 3, 6 and 8. All experiments were performed in triplicate and results are expressed as the average number of cells per microscopic field. (**C**) After culture for 8 days, melanocytes were harvested using trypsin and the number of melanocytes prepared using the conventional method and the new method were counted using a cell counting plate. The number of cells was calculated from triplicate experiments, and the results show the average number of cells per 100 mm dish. (**D**) After counting the number of melanocytes using an automated cell counter, melanocytes prepared using the conventional method or the new method were seeded in new dishes and microscopic photos were taken at day 9. (**E**) Melanocytes isolated using the new method at passage 0 or at passage 1 were fixed and stained with Ki67 antibody (red) and DAPI (nuclei, blue). (**B-C**), Student's *t*-test was used to analyze differences between the new and the conventional method, ** $P < 0.01$, *** $P < 0.005$. All scale bars represent 100 μm.

Figure 3. Y-27632 enhances melanocyte growth through regulation of keratinocytes. (**A**) Pure melanocytes (passage 3) were treated with 10 μM Y-27632 for 12, 24, 48, and 60 h and the number of melanocytes was analyzed by CCK-8 assay. (**B**) Pure melanocytes (passage 3) were treated with 10 μM Y-27632 for 48h and then were fixed for Ki67 staining. Quantification of percentage of Ki67 positive cells among total live cells indicated with DAPI nuclear staining. (**C-D**) Pure melanocytes (passage 3) were treated with 10 μM Y-27632 for 48 h and then were either stained for apoptotic cells with Annexin V-FITC and propidium iodide followed by FACS analysis (**C**) or fixed for TUNEL assay. (**E**) Isolated epidermis after trypsin digestion was plated with TIVA medium with or without Y-27632 (10 μM) and 2 days later immunofluorescence analysis of keratinocytes was performed with a pan-cytokeratin antibody (pan-ck). (**F**) Quantification of keratinocytes in the Y-27632-treated and control dishes from (**E**) as percentage of keratinocytes in a total of 500 cells counted. (**G**) Pure passage 3 keratinocytes were seeded in TIVA medium with or without Y-27632 (10 μM). Images of epidermal cells are shown at one day after seeding. (**H**) Quantification of attached keratinocytes in the Y-27632-treated and control dishes from (**G**) as the percentage of attached cells in a total of 5000 cells seeded. (**I**) Pure passage 3 keratinocytes were plated with TIVA medium in the presence or absence of Y-27632. The

microscopic images were obtained at 24 h. (**J**) Left panel: Equal numbers of pure passage 3 melanocytes were plated with TIVA medium in 4 groups with addition(s) as indicated: 1) Y-27632 (10 μM) alone (Y in graph), 2) passage 3 keratinocytes alone , 3) keratinocytes and Y-27632 (10 μM) (K+Y), and 4) no addition (negative control, con). Relative fold changes in melanocyte proliferation compared to the negative control were determined by counting the number of melanocytes at 24 h and 48 h after plating. Right panel: Conditioned TIVA media were obtained from the 4 groups of passage 3 melanocyte cultures in (left panel) at 48 h after plating. Then, equal numbers of passage 3 melanocytes were plated in 4 groups, each with one of the conditioned media. Relative fold changes in melanocyte proliferation compared to the negative control were determined by counting the number of melanocytes at 24 h and 48 h after plating. (**A-J)** All experiments have been repeated for 3 times, *p<0.05, **p<0.01, ***p<0.005. All Bars in the images represent 100 μm. The figure has been modified from Mi et al.[16].

Figure 4: Characterization of melanocytes isolated using the new method. (**A**) Representative images of immunofluorescence staining of MITF (red) in melanocytes prepared using the new or the conventional method. DAPI stains nuclei (blue). (**B**) Cell pellets were collected from melanocytes prepared using the new method and were treated for 2 days with forskolin (FSK) or DMSO vehicle as a control (con). All scale bars represent 100 μm.

DISCUSSION:

The protocol described here was based on a recent publication[16]. Some attention should be paid to the following critical steps to achieve the best results with the new method. First, successful separation of the epidermis and the dermis is crucial. Cut the adult foreskin tissues into 3-4 mm wide strips using a scalpel blade to make the dispase work more thoroughly and easily to separate the epidermis from the dermis. Second, when separating those two layers, contamination of the dermis should be avoided since dermal fibroblasts can also grow in melanocyte culture medium. Third, the trypsin digestion step should be less than 30 min; otherwise, it will significantly decrease the viability of the isolated cells.

This new method significantly shortens the time needed for melanocyte isolation. Human epidermal melanocytes produce melanin, which protects the skin from solar irradiation damage. Eisinger and Marko proposed the first practical method for the culture of human melanocytes from the epidermis[5]. Most importantly, the tissues used usually come from newborn babies and it is very difficult to isolate primary melanocytes from adult skin tissues. The Rho-associated protein kinase inhibitor Y-27632 significantly improves the efficiency of epidermal stem cell isolation[11-13]. Additionally, we also reported a method for isolating human primary epidermal cells from skin tissues in the presence of Y-27632[10-11]. In this protocol, Y-27632 was shown to efficiently increase the yield of primary melanocytes. Using the conventional method has a low cell recovery rate and needs around 3 to 4 weeks of culture for melanocytes to grow in sufficient numbers to passage. This new method, in which Y-27632 is added to the TIVA growth medium, increased melanocyte yield by about five times, and the melanocytes obtained can proliferate normally. We also tested the new method using a commercial medium and found similar results. Regarding the mechanism involved, a recent study demonstrated that the effect of Y-27632 to enhance the efficiency of melanocyte isolation occurs mainly through keratinocytes[16].

Importantly, we obtained pure melanocytes with normal function using the new method. The Passage 1 (P1) melanocytes were nearly all stained for MITF expression indicating that pure melanocytes are obtained after the first passage. Primary melanocytes with high growth potential are very important for biological research and clinical applications. The melanocytes isolated using the new method can be induced to pigment after treatment with forskolin, an activator of adenylate cyclase that increases cyclic AMP levels, indicating these melanocytes can function normally, which will be useful for studying pigmentation defects and melanoma[14].

In summary, a highly efficient method was successfully established to isolate primary melanocytes from adult skin tissues. This improved method could contribute to provide sufficient numbers of melanocytes in a rapid manner for biological research and for clinical applications.

ACKNOWLEDGMENTS:

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DISCLOSURES:

All authors declare no interest of conflict.

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Figure 1

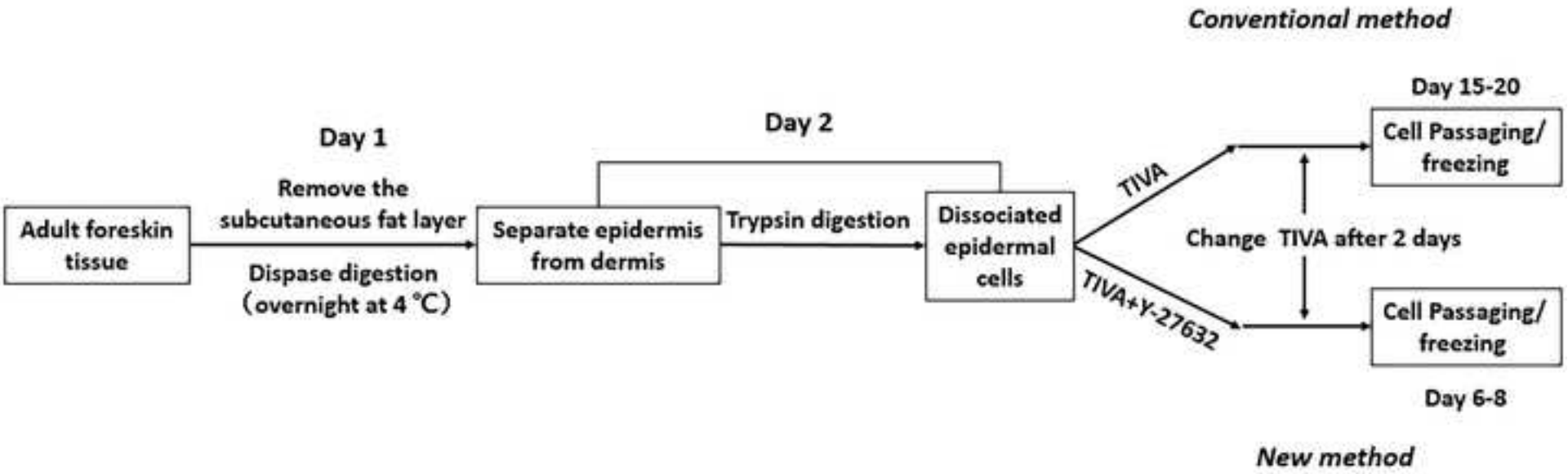

Figure 2

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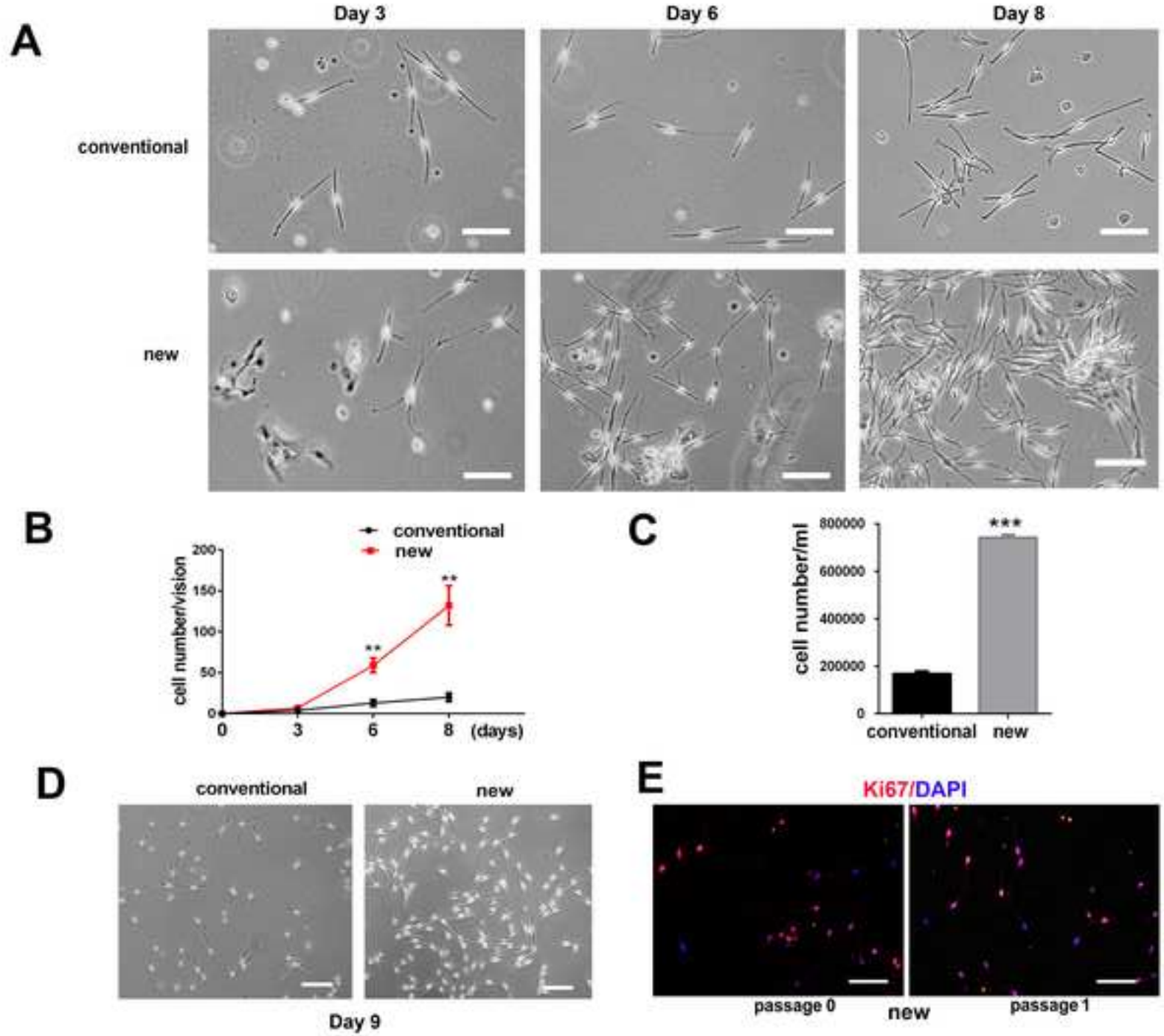


Figure 3

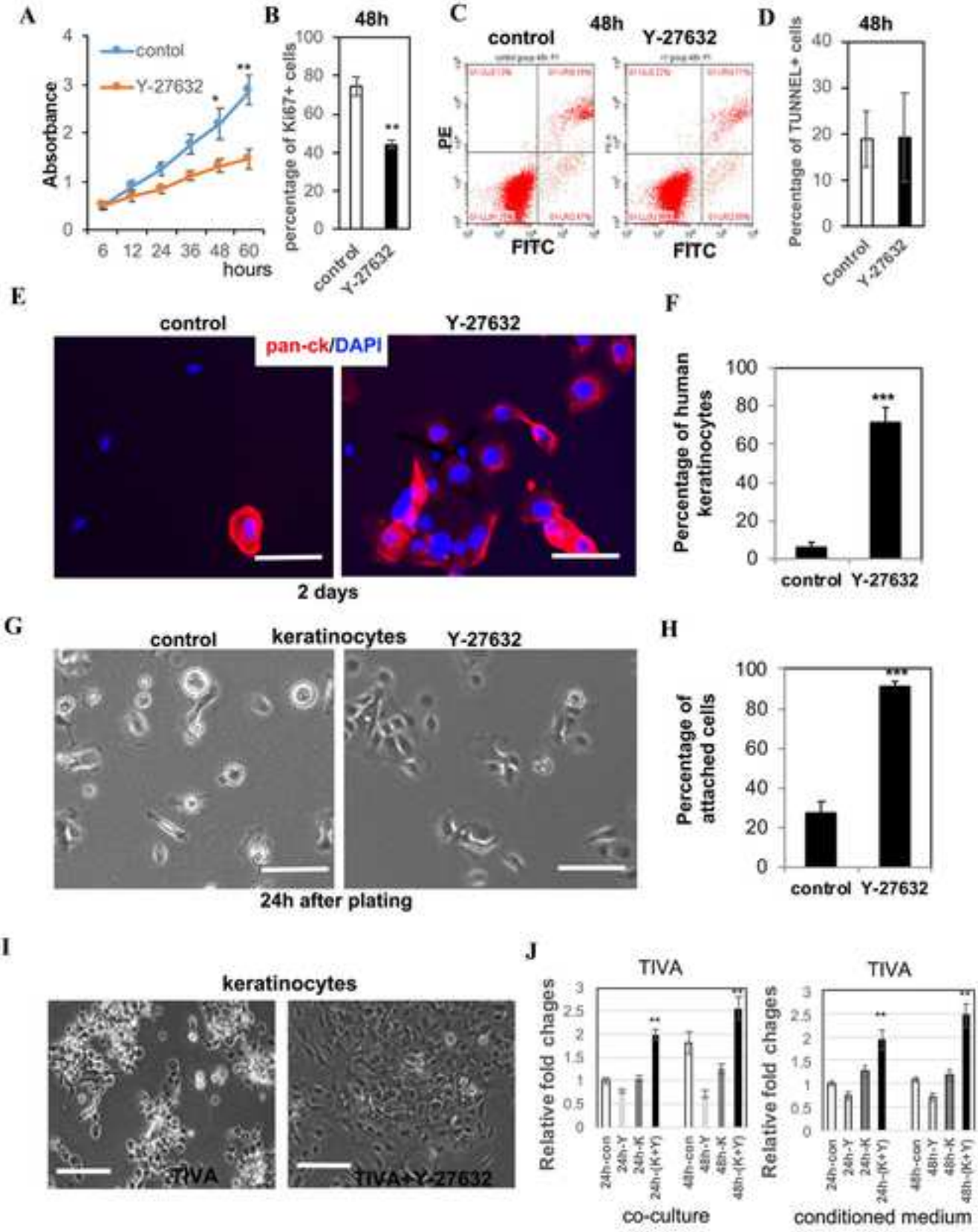
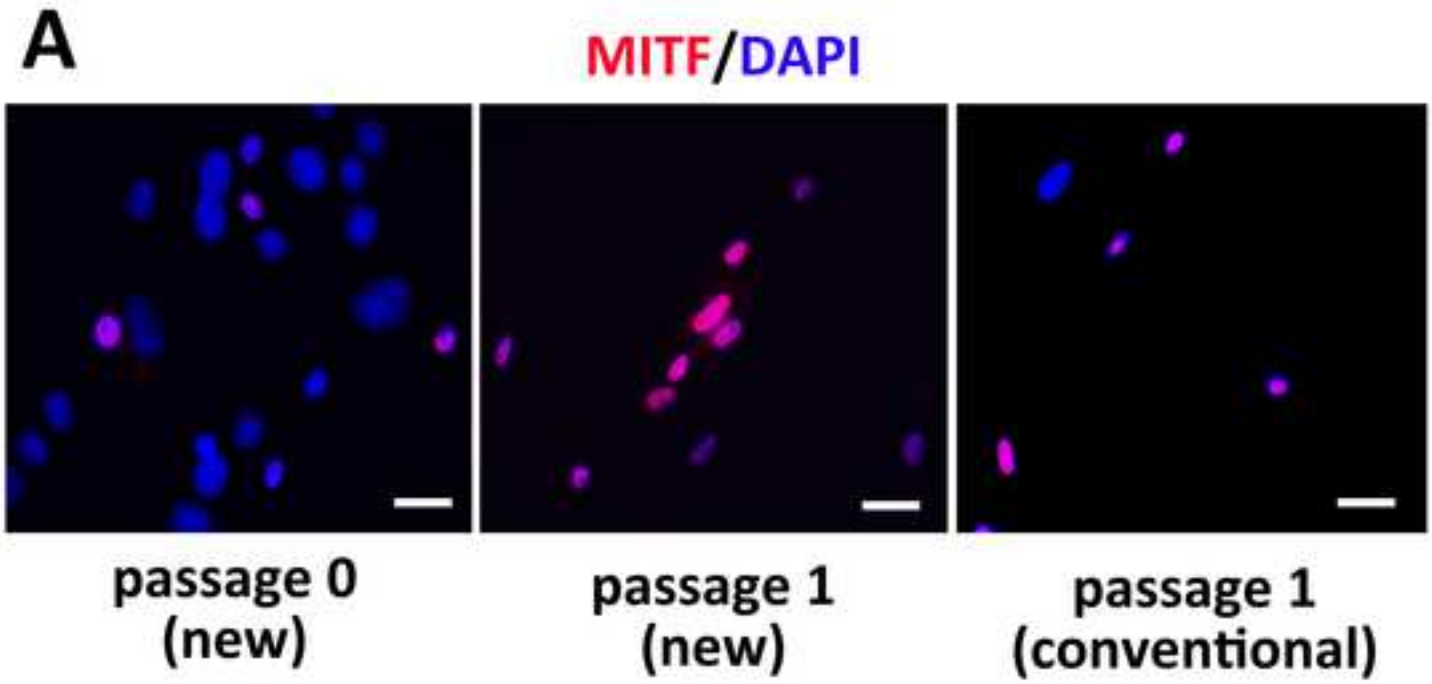


Figure 4



MATERIALS AND EQUIPMENT

Y-27632 Enriches the Yield of Human Melanocytes from Adult Skin Tissues

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Alexa fluor-594 donkey anti-mouse IgG	Thermo Fisher Scientific	A21203	For Immunofluorescence
Alexa fluor-594 donkey anti-rabbit IgG	Thermo Fisher Scientific	R37119	For Immunofluorescence
Cell Culture Dish	Eppendorf	30702115	For cell culture
Cell Strainer	Corning incorporated	431792	Cell filtration
15 mL Centrifuge Tube	KIRGEN	171003	For cell centrifuge
50 mL Centrifuge Tube	KIRGEN	171003	For cell centrifuge
CO_2 Incubator	Thermo Scientific	51026333	For cell incubating
Constant Temperature Shaker	Shanghai Boxun	150036	For water bath
DAPI	Abcam	ab104139	For Immunofluorescence
Dispase	Gibco	17105-041	For melanocyte isolation
DMEM	Thermo Scientific	C11995500	Component of neutralization medium
Fetal Bovine Serum	Biological Industries	04-001-1AC5	Component of neutralization medium
Fluorescence microscope	Olympus	5E44316	For Immunofluorescence
Forskolin	MCE	HY-15371	Induce pigmentation
Glutamine	Thermo Fisher Scientific	25030081	melanocyte culture medium
Ham's F12	Thermo Scientific	11330032	melanocyte culture medium
Inverted microscope	Olympus	5C42258	For cell microscopic observation
3-isobutyl-1-methyl xanthine (IBMX)	Sigma	17018	melanocyte culture medium
mouse anti-human MITF	Abcam	ab12039	For Immunofluorescence
Na_3VO_4	Sigma	S6508	melanocytes culture medium
N6,2'-O-dibutyryladeno-sine 3',5'-cyclic monophosphate (dbcAMP)	Sigma	D0627	melanocyte culture medium
12-O-tetradecanoyl phorbol-13-acetate (TPA）	Sigma	79346	melanocyte culture medium
Penicillin Streptomycin	Thermo Scientific	15140-122	Antibiotics
rabbit anti-human Ki-67	Abcam	ab15580	For Immunofluorescence
Phosphate buffered solution	Solarbio Life Science	P1020-500	Washing solution
Sorvall ST 16R Centrifuge	Thermo Scientific	75004380	Cell centrifugation
TC20TM automated cell counter	Bio-Rad	1450102	Automatic cell counting
0.05% Trypsin	Life Technologies	25300-062	For melanocyte dissociation
Y-27632	Sigma	Y0503	For melanocyte isolation

May 30, 2020

Re: JoVE61226R2

Dear Dr. Nguyen,

Appended is a revised version of our manuscript titled “Y-27632 Enriches the Yield of Human Melanocytes from Adult Skin Tissues” together with our responses to the editorial comments.

On behalf of our co-authors, we thank you again for all constructive comments and suggestions to improve our manuscript. We responded to each comment and suggestion point by point as following, and suggested, we added a new figure (Figure 3), which modified from previous published figures (included the permission from PCMR), and we made corresponding changes in both manuscript and video. And all changes in the revised manuscript were marked with underlying.

We hope that you will find the revised version now suitable for publication in ***Journal of Visualized Experiments.*** We deeply appreciate your consideration, and we are looking forward to your positive response.

Sincerely,

Xunwei Wu, M.D, Ph.D

REPONSES To Editorial COMMENTS

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:
1. Please include some figures from your previous publication (Reference 16) with permission to further strengthen the protocol.

RESPONSES: Thanks for your suggestion. We added a new figure, Figure 3 in the revised

version, which has been modified from previous published figures with the permission.

Changes to be made by the Author(s) regarding the video:

1. 02:46 & 04:38 - Centrifuge at 1000 gram per minute for five minutes"" should be ""Centrifuge at 1000 times g per minute for five minutes"". They re-recorded this line but left out the ""g"", making it ""Centrifuge at 1000 times for five minutes"". If that ""g"" is critical, ask them to rerecord one more time and to vocalize the ""g"" in ""times g"".
• 04:38 Same thing here with the dropping of the ""g"""

RESPONSES: Thanks for your suggestion. We made the change correspondingly: "Centrifuge at 200 times g for five minutes". Since the centrifuge speed is 1000 rpm (around 200xg).

2. 03:50 The sides of the frame are white where in the rest of the video they are black during the microscope segments. Consider making these margins also black.

RESPONSES: Thanks for your recommendation. We changed the margins which showed the under-microscope view on day 9 to black.

3. 04:33 The footage restarts here. You can slow the clip down or create a freeze frame if you'd like to emphasize it, but don't loop it like this by starting it over.

RESPONSES: Thanks for your proposal. We extended the time of a single frame and removed the duplicated clip.

4. 7:31: Please identify all on screen talent with their name.

RESPONSES: Thanks for your advice. We added the name into the video accordingly as suggested.

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