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Original Article

Modified human skin cell isolation protocol and its influence on keratinocyte and melanocyte culture

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ABSTRACT

Introduction: With the increasing emphasis on the use of nonanimal ingredients in clinical care, studies have proposed the use of TrypLE™ as an alternative to trypsin. However, previous research has reported insufficient cell yield and viability when using TrypLE to isolate skin cells compared to the dispase/trypsin-EDTA method. This study aimed to propose an improved method for increasing the yield and viability of cells isolated by TrypLE and to evaluate isolated keratinocytes and melanocytes.
Methods: Foreskin tissues were isolated to keratinocytes and melanocytes using the trypsin-EDTA protocol and our modified TrypLE protocol. The yield and viability of freshly isolated cells were compared, the epidermal residue after cell suspension filtration was analyzed histologically, and the expression of cytokeratin 14 (CK14) and Melan-A was detected by flow cytometry. After cultivation, keratinocytes and melanocytes were further examined for marker expression and proliferation. A coculture model of melanocytes and HaCaT cells was used to evaluate melanin transfer.
Results: The yield, viability of total cells and expression of the keratinocyte marker CK14 were similar for freshly isolated cells from both protocols. No differences were observed in the histologic analysis of epidermal residues. Moreover, no differences in keratinocyte marker expression or melanocyte melanin transfer function were observed after culture. However, melanocytes generated using the TrypLE protocol exhibited increased Melan-A expression and proliferation in culture.
Conclusion: Our TrypLE protocol not only solved the problems of insufficient cell yield and viability in previous studies but also preserved normal cell morphology and function, which enables the clinical treatment of depigmentation diseases.

1. Introduction

Commercially immortalized cell lines are commonly used in medical research due to their cost-effectiveness, unlimited replication capabilities, and ease of manipulation [1]. However, it is important to note that cell lines are genetically modified and transformed, which alters their physiological properties and renders them unrepresentative of in vivo conditions [2]. Human primary cells are becoming increasingly important in cell and molecular biology because they provide excellent model systems for studying the normal physiology and biochemistry of cells. They are also a mainstay of clinical cell therapy [3]. The isolation of primary cells from biopsy tissue is a crucial step for this purpose.

Abbreviations: CK14, cytokeratin 14; FBS, fetal bovine serum; PBS, phosphate buffered saline; TESSs, tissue-engineered skin substitutes; DMEM, dulbecco's modified eagle medium; FVS450, fixable viability stain 450; BSA, bovine serum albumin; MITF, microphthalmia-associated transcription factor; TRP1, tyrosinase-related protein-1; CK19, cytokeratin 19; TYR, tyrosinase; DCT, dopachrome tautomerase.

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Human primary epidermal cells were isolated through enzymatic digestion and cultured with appropriate growth media. The classic two-step enzymatic digestion method involves submerging the skin in dispase solution at 4 °C overnight. Then, the epidermis was mechanically separated from the dermis, and the epidermis was submerged in trypsin solution at 37 °C [4]. Trypsin is a digestive enzyme that is commonly used in cell culture and for isolating cells from the epidermis [5]. It is derived mainly from pig pancreas and carries the risk of contamination with swine-borne viruses [6]. Furthermore, fetal bovine serum (FBS) was utilized to inactivate trypsin. However, it may contain animal viruses that can lead to disease transmission, such as bovine spongiform encephalopathy [7]. Consequently, it is preferable to use nonanimal ingredients in clinical applications [8].

In recent years, TrypLE has been proposed as an alternative to trypsin. TrypLE is a gentler xeno-free enzyme produced by recombinant technology that can be inactivated by dilution with phosphate buffered saline (PBS) [9]. Lagerwall et al. [10] compared the efficiency of trypsin and TrypLE in digesting the same area of dermatomes for the same duration. The viability of cells isolated by TrypLE, as well as the expression of CK14 in keratinocytes, was significantly lower than that of cells isolated by trypsin. Laura Frese et al. [11] observed that TrypLE isolated fewer live cells than trypsin. The method for isolating human epidermal cells must be robust, standardized, and yield viable cells to ensure efficient treatment of large areas of trauma with minimal donor skin [12]. Thus, further refinement is necessary for epidermal cell isolation using TrypLE.

The manufacture of tissue-engineered skin substitutes (TESSs) for clinical treatments, such as burn and vitiligo treatment [13], typically involves the isolation of autologous keratinocytes and melanocytes to promote wound repair and pigment recovery [14,15]. Due to the limited size of donor skin, the protocol for cell isolation must ensure adequate cell yield and viability while preserving the biological properties of the cells.

The aim of this study was to propose a protocol for cell isolation that prolongs TrypLE digestion time and increases physical manipulation. This approach provides isolated cell yield, viability, and expression of relevant markers at levels comparable to those of the conventional trypsin protocol. Further investigation of the effect of the protocol on cell culture will enable the clinical application of TrypLE.

2. Materials and methods

2.1. Culture, apoptosis assay and CCK-8 assay of cell lines

2.1.1. Cell culture

HaCaT (Cas9X™, Suzhou, China) and PIG1 (Ybio, Shanghai, China) cells were cultured in dulbecco's modified eagle medium (DMEM) (Gibco®, USA) supplemented with 10% FBS (ShuangRu Biotech, Suzhou, China), 100 U/mL penicillin, and 100 μg/mL streptomycin (ShuangRu Biotech, Suzhou, China). All cells were incubated at 37 °C with 5% CO_2.

2.1.2. Apoptosis assay by flow cytometry

Flow cytometry was used to demonstrate the ability of the tested compounds to induce apoptosis. HaCaT and PIG1 cells were incubated with TrypLE Express (1 ×) (Gibco, USA) at 37 °C for 10, 20, 30, 45, or 60 min. The adherent cells subjected to normal digestion were used as the control group. TrypLE Express (1 ×) was deactivated with PBS. The cells were then harvested and resuspended in PBS. The cells were then stained with a solution of Annexin V-FITC (5 μl)/propidium iodide (PI) (10 μl) (Multi Sciences, China). Flow cytometric analysis was performed after the drug-cell mixture was incubated for 5 min in a dark room using a flow cytometer (Beckman Coulter, USA).

2.1.3. CCK-8 assay

The viability potential of HaCaT and PIG1 cells was evaluated by the CCK-8 method (Dojindo, Japan). HaCaT and PIG1 cells were incubated with TrypLE Express (1 ×) at 37 °C for 60 min. The process of normal digestion of adherent cells was used for the control group, which were harvested by incubation with trypsin-EDTA (New Cell & Molecular Biotech, China) solution for 3 min at 37 °C. The cells (5×10^3/well) were then seeded in a 96-well plate (Corning, USA) with 10 μL of CCK-8 solution. The cells were then cultured for 2 h, and the absorbance at 450 nm was measured using a microplate reader (Molecular Devices, USA).

2.2. Primary cell isolation and culture

Human keratinocytes and melanocytes were obtained from three human adult discarded skin biopsies (foreskin (n = 3)) from healthy males (23, 24, and 29 years old). Adipose tissue and blood traces were removed prior to the initiation of the two isolation protocols.

2.2.1. Our modified protocol (TrypLE protocol) is shown in Fig. 1

Biopsies were incubated in 5 mg/ml dispase II (Sigma–Aldrich, USA) at 4 °C overnight (15 h). The epidermis was separated from the dermis with fine forceps. The isolated epidermal layers were placed in TrypLE Express (1 ×) solution for 40–50 min at 37 °C on a constant temperature shaker at 40 RPM. The cell suspension was then neutralized with PBS, gently aspirated, filtered through a 70 μm cell strainer (Labgic, Beijing, China), and centrifuged (5 min/300×*g*).

2.2.2. Control protocol (trypsin-EDTA protocol) is shown in Fig. 1

Biopsies were incubated in 5 mg/ml dispase II at 4 °C overnight (15 h). The epidermis was separated from the dermis with fine forceps. Isolated epidermal layers were placed in trypsin-EDTA solution for 25 min at 37 °C. The cell suspension was then neutralized with DMEM containing 10% FBS, filtered through a 70 μm cell strainer, and centrifuged (5 min/300×*g*).

2.2.3. Primary cell culture

Keratinocytes were counted and seeded at 40,000 cells/cm^2 in dermaCult™ keratinocyte expansion basal medium (Stemcell, Canada) supplemented with dermaCult keratinocyte expansion supplement (50 ×). Melanocytes were counted and seeded at 160,000 cells/cm^2 in melanocyte medium (MelM, ScienCell, USA) supplemented with melanocyte growth supplement (MelGS), fetal bovine serum, and penicillin/streptomycin solution. The culture medium was changed after 72 h and then every 2 days. Using the extracellular matrix component collagen I (Solarbio, Beijing, China) as a matrix, the cells were maintained at 37 °C with 5% CO_2.

2.3. Primary cell viability and yield

Freshly isolated cells were stained with 0.4% trypan blue (1:1) and counted using an automated cell counter (Invitrogen Countess 3, Thermo Fisher Scientific, USA) with parameters between 7 μm and 21 μm. The viable cell yield per cm^2 was calculated from the total number of cells and the area of each human skin sample.

2.4. Hematoxylin-eosin (HE) staining

After 4% paraformaldehyde fixation, alcohol dehydration and paraffin embedding, the filtered epidermal residues of the cell

suspensions isolated by the two protocols were serially sectioned. The sections were then mounted on slides and stained with hematoxylin and eosin (Servicebio, Wuhan, China).

2.5. Characterization of isolated keratinocytes and melanocytes by flow cytometry

Flow cytometric analysis was used to determine the percentage of isolated cells. To detect keratinocytes, cells were stained with recombinant anti-cytokeratin 14 (CK14) antibody (ab210414, Abcam, United Kingdom) conjugated with phycoerythrin (PE) diluted 1:200. To detect melanocytes, the cells were stained with the recombinant anti-Melan-A antibody Alexa Fluor 647 (ab225500, Abcam, United Kingdom) at a dilution of 1:200. To detect viability, the cells were stained with fixable viability stain 450 (FVS450) (BD Biosciences, USA) at a 1:200 dilution.

2.6. Immunocytochemistry

Passage 2 keratinocytes and melanocytes were grown in 6-well plates at a density of 50,000 cells/well. After 24 h, the cells were fixed with 4% polyformaldehyde (10 min), permeabilized with 0.3% Triton X-100 for 10 min, and then blocked with 3% bovine serum albumin (BSA) for 1 h at room temperature. Keratinocytes were incubated with recombinant anti-p63 antibody (ab124762, Abcam, United Kingdom) at a 1:200 dilution overnight at 4 °C, followed by further incubation at room temperature for 2 h with donkey anti-rabbit IgG (Alexa Fluor® 488) secondary antibody (A-21206, Thermo Fisher Scientific, USA) at a 1:200 dilution. Melanocytes were then incubated with recombinant anti-microphthalmia-associated transcription factor (MITF) (ab3201, Abcam, United Kingdom) and anti-tyrosinase-related protein-1 (TRP1) (ab178676, Abcam, United Kingdom) antibodies at a 1:200 dilution overnight at 4 °C, followed by further incubation at room temperature for 2 h with a goat secondary antibody to mouse IgG (Alexa Fluor 594) (A-11005, Thermo Fisher Scientific, USA) and a donkey secondary antibody to rabbit IgG at a 1:200 dilution. Nuclear DNA was labeled blue with 4′,6-diamidino-2-phenylindole (DAPI) (Beyotime, Shanghai, China) under a fluorescence microscope.

2.7. Primary cell proliferation assay

The viability potential of keratinocytes and melanocytes was evaluated using the CCK-8 method. Keratinocytes and melanocytes (5×10^3/well) at passage 2 were seeded in a 96-well plate and stimulated with 10 μL of CCK-8 solution. The cells were then incubated for 2 h, after which the absorbance at 450 nm was measured using a microplate reader.

2.8. Evaluation of melanin transfer from melanocytes

Melanocytes isolated by both protocols and HaCaT cells were inoculated into 6-well plates at a 1:2 ratio, and both cell culture media were added to the well plates at a 1:2 ratio. The control group consisted of HaCaT cells cultured alone. After 3 days, the cells were fixed with 4% polyformaldehyde (10 min), permeabilized with 0.3% Triton X-100 for 10 min, and then blocked with 3% BSA for 1 h at room temperature. The cells were then incubated with recombinant anti-cytokeratin 19 (CK19) (ab7754, Abcam, United Kingdom) and anti-TRP1 antibodies at a 1:200 dilution overnight at 4 °C, followed by further incubation at room temperature for 2 h with a goat secondary antibody to mouse IgG and a donkey secondary antibody to rabbit IgG at a 1:200 dilution. Nuclear DNA was labeled blue with DAPI under a fluorescence microscope.

2.9. Statistical analysis

GraphPad Prism 10 and FlowJo v10 were used for statistical analysis and graphing. Then, depending on the comparison, one-way ANOVA and Tukey's multiple comparison test or multiple t tests were used to analyze the results. All values are presented as the mean ± standard deviation (SD). A p value less than 0.05 was considered to indicate statistical significance.

3. Results

3.1. The incubation of cells with TrypLE for 60 min had no effect on cell viability, so the digestion time could be extended accordingly

It is important to consider whether enzyme digestion time affects cell viability when isolating primary epidermal cells. The results of the apoptosis assay after incubation of HaCaT and PIG1 cells with TrypLE showed that there was no statistically significant difference in the effect of incubating the cells with TrypLE for up to 60 min on cell viability compared to that of the control group (shown in Fig. 2a–d). In addition, the CCK-8 assay showed that the proliferation of cells after 60 min of TrypLE incubation was significantly greater than that of control cells incubated with trypsin-EDTA for 3 min (shown in Fig. 3a–b). The above results indicate that incubation of cells with TrypLE has no effect on cell viability for up to 60 min, so the digestion time can be extended accordingly but does not exceed 60 min when the cells are isolated.

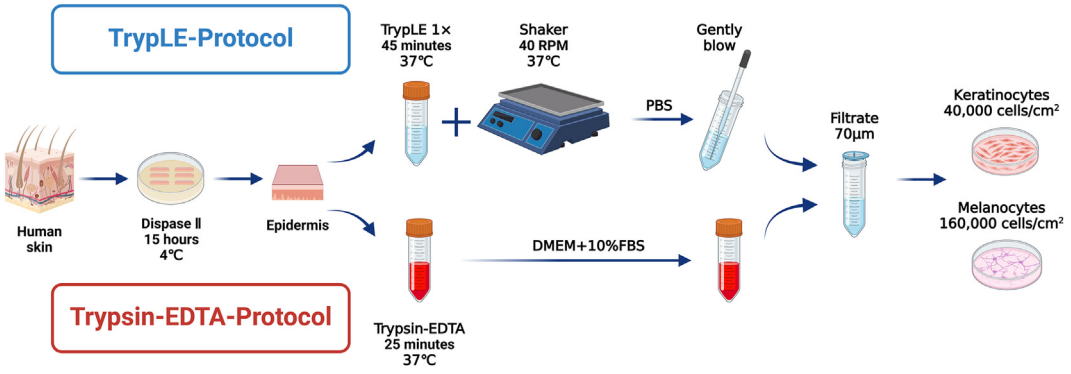

Fig. 1. Schematic of the two isolation protocols created using BioRender.com.

3.2. There was no significant difference in the viability or yield of freshly isolated cells between the two protocols

Cell viability and yield are the most important characteristics of cell therapy products. There was no significant difference in cell viability or yield per cm^2 of skin between freshly isolated cells from the trypsin-EDTA protocol and those from the TrypLE protocol (shown in Fig. 4b–c). In addition, HE staining showed that there was little visible cellular residue in the epidermal residue after filtration of both protocols, only the stratum corneum (shown in Fig. 4a), indicating that the cells were largely separated. The above results indicate that our improved TrypLE protocol has solved the problem of insufficient cell viability and yield reported in previous studies.

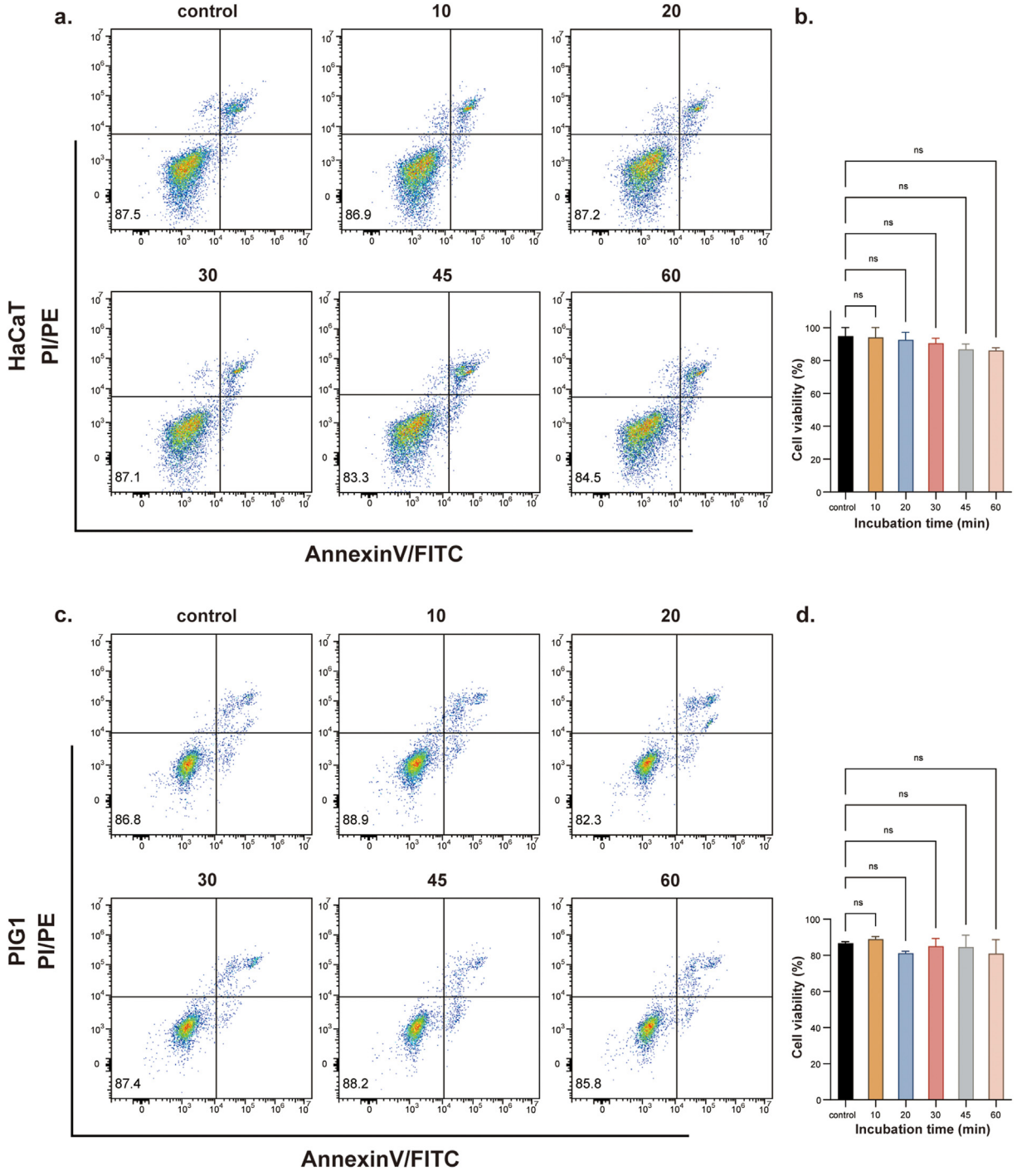

Fig. 2. Effect of TrypLE incubation for different durations on cell viability. (a) Representative images of flow cytometry apoptosis assays for HaCaT cells incubated with TrypLE for different durations. (b) Comparison of HaCaT cell viability after incubation for different durations. (c) Representative images of flow cytometry apoptosis assays for different durations of TrypLE incubation with PIG1. (d) Comparison of PIG1 cell viability after incubation for different durations. The control refers to the enzymatic digestion of adherent cells. Compared with those in the control group, the differences in HaCaT and PIG1 cell viability (lower left quadrant of the flow cytometry result graph) after TrypLE incubation for different durations (10, 20, 30, 45, or 60 min) were not statistically significant ($p > 0.05$). The data are presented as the means ± SDs; ns: differences were not statistically significant.

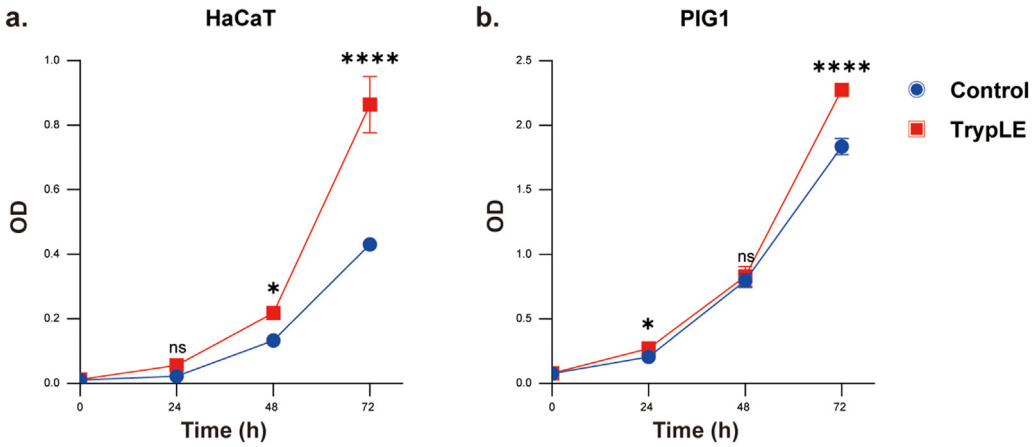

Fig. 3. Effect of incubation with TrypLE for 60 min on cell proliferation. (a) Proliferation of HaCaT cells after TrypLE incubation. (b) Proliferation of PIG1 cells after TrypLE incubation. The control refers to the normal digestion of adherent cells using trypsin-EDTA. After 72 h, HaCaT and PIG1 cell proliferation was greater in the CCK-8 assay group than in the control group ($p < 0.0001$). The data are expressed as the means ± SDs; ns: not statistically significant, *: $p < 0.05$, ****: $p < 0.0001$.

3.3. *Freshly isolated and cultured keratinocytes from both protocols were characterized, including cell marker expression and proliferation*

The flow cytometry results showed that the level of FVS450 negativity was similar in freshly isolated cells from both protocols (shown in Fig. 5a–b), indicating that there was no significant difference in cell viability. Moreover, the difference in CK14 expression levels was not statistically significant (shown in Fig. 5a–c). After culture and passage, immunocytochemical staining showed that the cells were both positive for P63 expression (shown in Fig. 5e). Furthermore, the CCK-8 assay results showed no statistically significant difference in the proliferation of keratinocytes (shown in Fig. 5d). The above results indicate that our enhancements did not alter keratinocyte biological properties.

3.4. *Freshly isolated and cultured melanocytes from both protocols were characterized for cell marker expression, proliferation, and melanin transfer*

The flow cytometry results showed that the level of FVS450 negativity was similar in freshly isolated cells from both protocols (shown in Fig. 6a–b), indicating that there was no significant difference in cell viability. However, the percentage of Melan-A-positive cells isolated by the TrypLE protocol (15.4 ± 0.3%) was significantly greater than that isolated by the trypsin-EDTA protocol (12.6 ± 0.6%) ($p < 0.01$) (shown in Fig. 6b and c). After culture and passage, immunocytochemical staining showed that the cells positively expressed both MITF and TRP1 (shown in Fig. 6e). In addition, the CCK-8 assay results showed that the proliferation of isolated melanocytes was significantly greater in the TrypLE protocol group than in the trypsin-EDTA protocol group ($p < 0.001$) (shown in Fig. 6d). Western blot analysis (shown in Supplementary Fig. 1) revealed that the expression of tyrosinase (TYR) was higher in the TrypLE protocol group than in the trypsin-EDTA protocol group ($p < 0.001$). Immunocytochemical staining of the coculture model showed that TRP1 was not expressed in HaCaT cells and that both groups of melanocytes synthesized and transported melanin to HaCaT cells (shown in Fig. 7). The aforementioned outcomes demonstrated that the improvement measures did not impact the typical functionality of melanocytes, including marker expression and melanin transfer. Moreover, the TrypLE protocol outperformed the trypsin-EDTA protocol in terms of melanin synthesis, cell yield and proliferation.

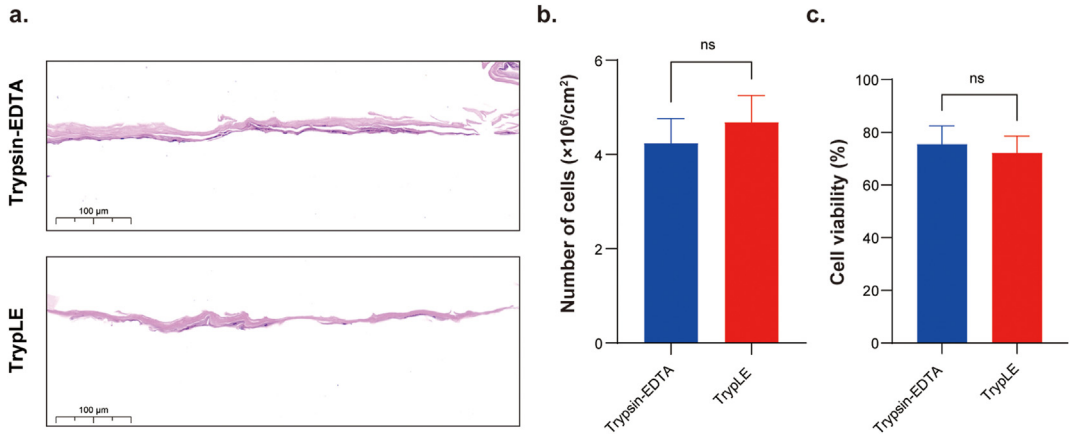

Fig. 4. Yield and viability of cells isolated by the TrypLE protocol and the trypsin-EDTA protocol. (a) Hematoxylin-eosin (HE) staining of epidermal debris after the isolation of cells by the two protocols revealed less cellular debris, scale bar: 100 μm. (b) Yield of cells isolated by the two protocols. (c) Viability of cells isolated by the two protocols. The difference in the yield and viability of isolated cells between the two protocols was not statistically significant ($p > 0.05$), and HE staining further confirmed thorough cell isolation. The data are expressed as the means ± SDs; ns: not statistically significant.

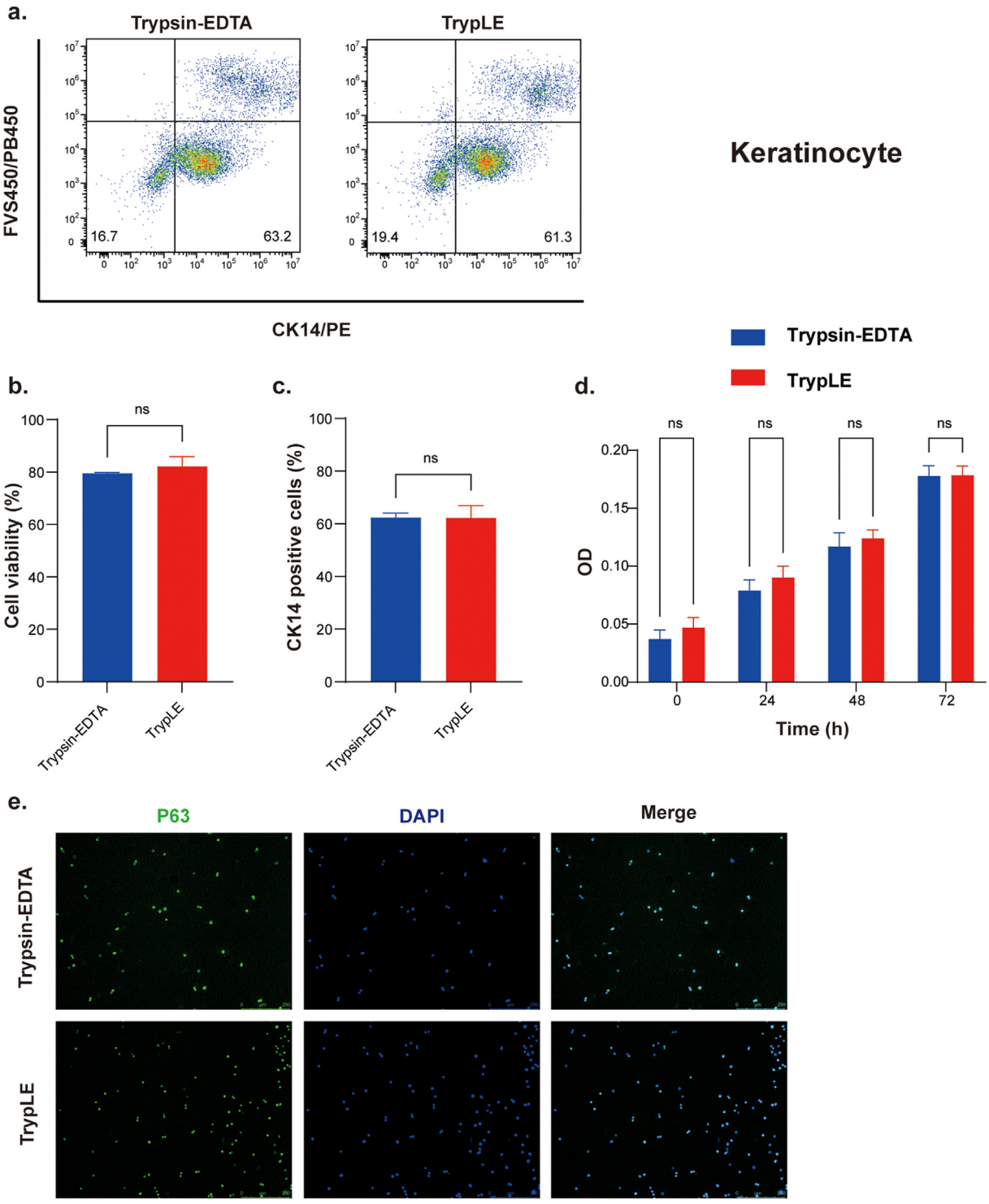

Fig. 5. Characterization of keratinocytes isolated by the TrypLE and trypsin-EDTA protocols. (a) Representative images of CK14 expression in freshly isolated keratinocytes from both protocols detected by flow cytometry, using FVS450 as a live-dead dye (negative for live cells) and CK14 as a keratinocyte marker. (b) Viability of cells isolated from both protocols detected by flow cytometry. (c) CK14 expression in cells isolated from both protocols detected by flow cytometry. (d) Comparison of proliferation of cells isolated from both protocols after culture. (e) Immunocytochemical staining of cells isolated from both protocols after culture, P63 (green) for keratinocyte marker and DAPI (blue) for nuclear marker, scale bar: 250 μm. Flow cytometry of freshly isolated keratinocytes from both protocols showed no statistically significant ($p > 0.05$) difference in keratinocyte CK14 expression levels (lower right quadrant) at comparable cell viability (sum of lower left and lower right quadrants). The difference in proliferation was not statistically significant ($p > 0.05$) according to the CCK-8 assay after cell culture, and immunocytochemical staining revealed that all keratinocytes expressed P63. The data are shown as the means ± SDs; ns: not statistically significant.

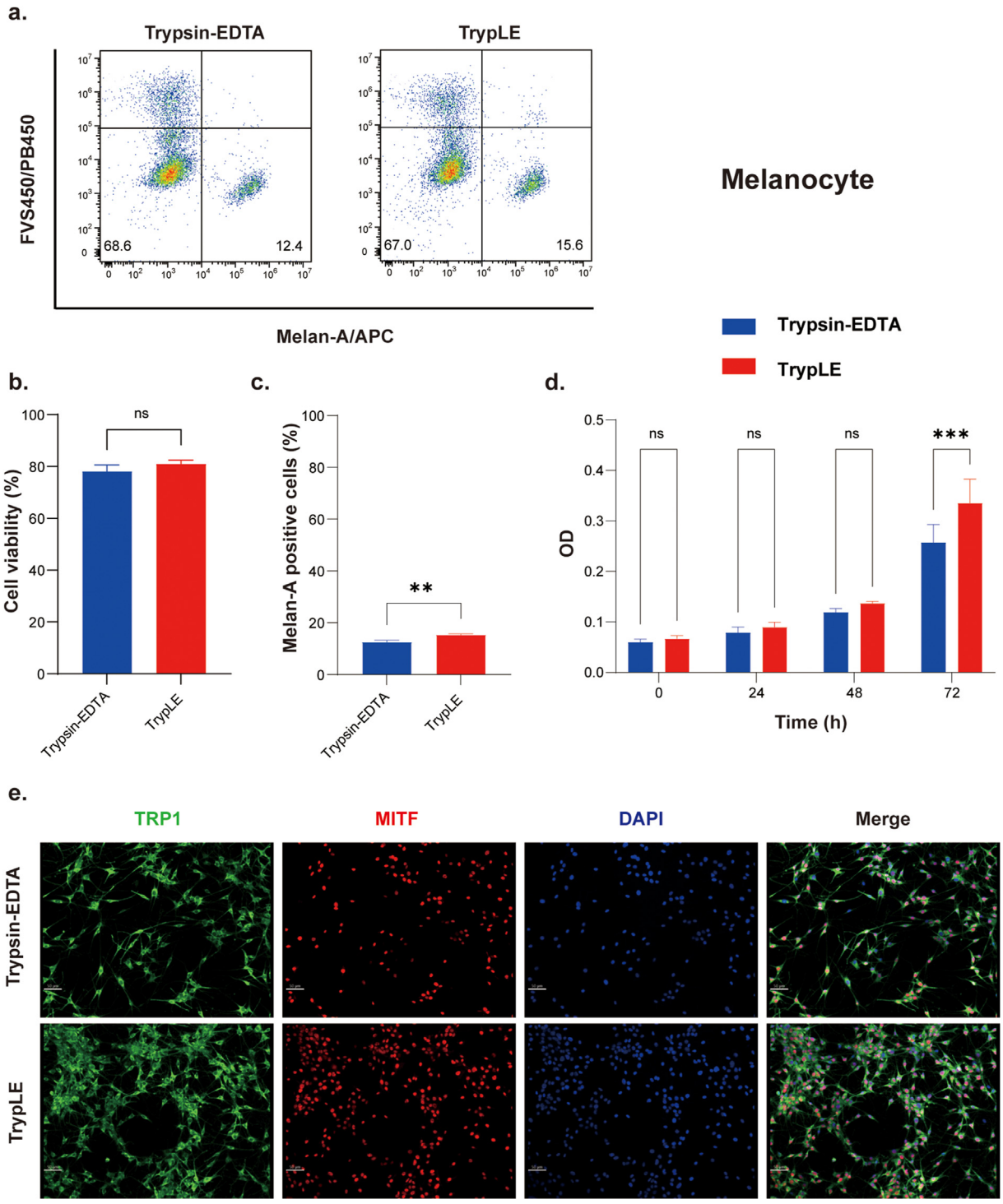

Fig. 6. Characterization of melanocytes isolated by the TrypLE and trypsin-EDTA protocols. (a) Representative images of Melan-A expression in freshly isolated melanocytes from both protocols detected by flow cytometry, using FVS450 as a live-dead dye (negative for live cells) and Melan-A as a melanocyte marker. (b) Viability of cells isolated from both protocols detected by flow cytometry. (c) Melan-A expression in cells isolated from both protocols detected by flow cytometry. (d) Comparison of proliferation of cells isolated from two protocols after culture. (e) Immunocytochemical staining of cells isolated from two protocols after culture, TRP1 (green) and MITF (red) for melanocyte labeling and DAPI (blue) for cell nucleus labeling, scale bar: 50 μm. A flow cytometry assay of freshly isolated melanocytes from both protocols showed that with comparable cellular viability (sum of the lower left and lower right quadrants), the melanocyte Melan-A expression level (lower right quadrant) was greater in the TrypLE protocol than in the trypsin-EDTA protocol (15.4 ± 0.3% vs. 12.6 ± 0.6%, $p < 0.01$). After cell culture, the proliferation of melanocytes in the 72-h CCK-8 assay was greater in the TrypLE protocol group than in the trypsin-EDTA protocol group ($p < 0.001$). Immunocytochemical staining showed that both TRP1 and MITF were expressed. The data are shown as the means ± SDs; ns: not statistically significant, **: $p < 0.01$, ***: $p < 0.001$.

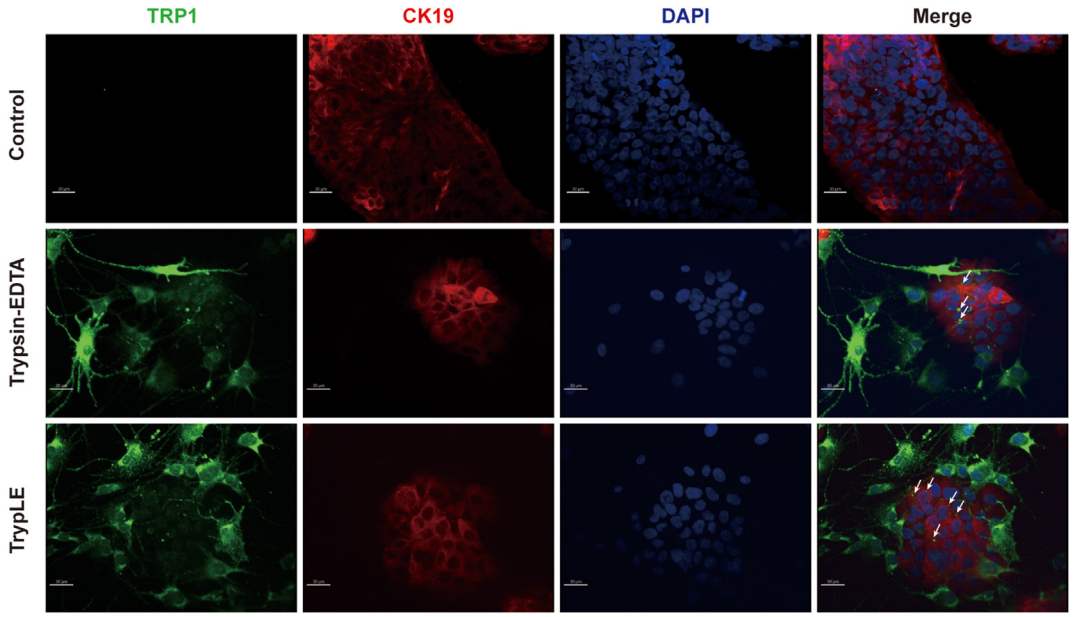

Fig. 7. Melanin transfer in cocultures of primary human melanocytes with HaCaT cells. TRP1 (green) is a melanosome marker, CK19 (red) is a HaCaT cell marker, and DAPI (blue) is a nuclear marker. The controls were HaCaT cells cultured alone. Immunocytochemical staining revealed that melanocytes isolated by both protocols synthesized melanin and transported it to HaCaT cells (indicated by white arrows in merged images), scale bar: 30 μm.

4. Discussion

In this study, we compared two human skin cell isolation protocols, the trypsin-EDTA protocol, which is the classical isolation protocol, and the TrypLE protocol, which is our optimized and improved protocol in response to the protocols of Cathrine Lagerwall [10] and Laura Frese [11] et al. Isolated melanocyte proliferation and Melan-A expression were greater when the TrypLE protocol was used. The other evaluated parameters measured were similar. First, no differences were observed in the yield and viability of freshly isolated cells from the two protocols. Second, none of the filtered epidermal debris HE stains showed significant cellular debris, indicating similar cell separation efficiencies. Third, the CK14 expression levels and proliferation of keratinocytes isolated from both protocols were similar. Fourth, immunocytochemical staining revealed that all keratinocytes expressed P63, and all melanocytes expressed MITF and TRP1. Finally, both types of melanocytes were able to synthesize and transfer melanin to HaCaT cells, indicating that both protocols preserved cell biology.

The main difference between the two protocols is in the second step: compared to the classical trypsin-EDTA protocol, our method uses TrypLE with a correspondingly longer digestion time and the addition of shaker mixing and a gentle blowing step before filtration. For TrypLE digestion at 37 °C, previous studies have reported that the reaction time ranged from 25 to 30 min [10,11]. By incubating HaCaT and PIG1 cells with TrypLE, we found that cell viability and proliferation were not affected within 60 min, so we decided to extend the digestion time of the enzyme. Increased shaking during digestion brings the enzyme into full contact with the skin and facilitates cell dissociation. In addition, a gentle blowing step prior to filtration was used because, in our preliminary experiments, we found flocculent residues in the cell filters, which may be one of the factors causing lower cell yield and viability. Our results showed no differences in cell viability and yield between the two protocols. Consistent with previous findings, none of the filtered epidermal debris showed significant cellular debris after histologic analysis. Undoubtedly, these improvements addressed the lack of cell viability and yield reported by Cathrine Lagerwall [10] and Laura Frese [11], demonstrating the effectiveness of our improved protocol.

Regarding the isolation of keratinocytes, cells isolated by both protocols showed similar results in terms of CK14 positivity. CK14 is one of the most commonly used markers for epidermal cells and is expressed mainly in the basal layer; its expression decreases as the cells migrate to the top of the epidermis and is absent in the stratum corneum [16]. Consistent with the results of our histologic analysis, the absence of significant cellular debris in the filtered epidermal debris explains the lack of difference in CK14 positivity. P63 is a specific surface marker for epidermal stem cells and plays a critical role in maintaining the biological properties, proliferation and differentiation of epidermal stem cells [17]. After culture and passage, immunocytochemical staining revealed that all keratinocytes expressed P63. In contrast, CCK8 assays showed no differences in keratinocyte proliferation. The above data indicate that our improved protocol achieves the levels of the classical trypsin-EDTA protocol and preserves the biological properties of keratinocytes.

Melanin, synthesized by melanocytes, protects skin from the UV light and determines skin, eye and hair color [18]. MITF is the major transcription factor involved in melanin synthesis and plays an important role in melanocyte survival, migration, proliferation and differentiation [19]. It initiates the synthesis of melanin by activating the transcription of pigment genes such as TYR, TRP1, and dopachrome tautomerase (DCT) [20]. TYR is a specific marker for melanocyte differentiation, catalysing the initial and rate-limiting step in the synthesis of melanin [21,22]. TRP1 is an enzyme required for melanin synthesis in melanocytes and plays a role in regulating the type and amount of melanin [23]. Immunocytochemical staining confirmed that the melanocytes isolated by both protocols expressed MITF and TRP1 and were able to synthesize melanin and translocate it to HaCaT cells. As a result, the two protocols have similar effects on the maintenance of the biological properties of melanocytes. More importantly, melanocytes isolated using our improved protocol exhibited increased Melan-A expression and proliferation. Western blot analysis revealed that the

expression of TYR was higher in the TrypLE protocol group. Our findings align with those of Shengyi Wang et al. [24], who observed that long-term trypsinization (2 h) resulted in a reduction in TYR, MITF expression and proliferation in melanocytes. They hypothesized that long-term incubation with trypsin causes dedifferentiation of mature melanocytes in vitro, which subsequently leads to a reduction in melanin synthesis. Although the trypsin digestion time in our experiments was less than 2 h, it also exhibited decreased melanocyte TYR expression. Since vitiligo treatment requires mature melanocytes with melanin synthesizing ability, our modified protocol has significant advantages. In addition, Yinjuan Wang [25] et al. suggested that the number of melanocytes isolated using TrypLE one-step enzymatic digestion and incubated at room temperature for 17 h was greater than that obtained via the trypsin-EDTA protocol, which is similar to our results. However, their protocol was only applied to a small sample of skin, and they did not perform melanocyte proliferation or melanin transport experiments, which shows that our protocol has a significant advantage.

The trypsin-EDTA protocol for isolation of cells from fresh skin tissue involves digestion of the epidermis with trypsin and inactivation of the enzyme with fetal bovine serum. Trypsin and fetal bovine serum are both of animal origin, and the safety of using animal-derived components to prepare cell products for the treatment of disease has been reported in recent years. A study by Tuschong et al. reported antibodies to fetal bovine serum in patients treated with in vitro cultured lymphocyte transplants. They also reported that anti-FCS IgG antibody levels remained elevated in patients who had received T-cell therapy for more than 8 years, despite washing of the cells prior to infusion [26]. For this reason, the use of products containing ingredients of animal origin should be avoided as much as possible in the preparation of cellular products. TrypLE is a recombinant enzyme without animal components that does not require fetal bovine serum and is inactivated by dilution with PBS [9]. Thus, our protocol improves upon TrypLE, making it better suited for clinical application.

5. Conclusion

This study reports an improved method for the isolation of keratinocytes and melanocytes from human skin using TrypLE. Compared with the established dispase/trypsin-EDTA protocol, the isolated melanocytes showed greater yield, less damage, greater cell proliferation after culture, and unchanged morphological and functional characteristics. The lack of cell yield and viability was addressed compared to previously reported isolation protocols using TrypLE. In addition, the significant advantage of our improved protocol is the use of products of nonanimal origin, which offers the possibility of treating pigmented diseases.

Ethics approval

The study protocol was reviewed and approved by the Changhai Hospital Ethical Review Authority (Shanghai, China) (reference number: CHEC2022-016) in conformity with the Declaration of Helsinki.

Authors' contributions

ZL, XF, XB and SX participated in the conception and design of the whole protocol. ZL, SJ, DC and HC were responsible for the experimental implementation. YW, ZY and CJ performed the data collection and analysis. ZL, SJ, DC and HC wrote this manuscript. XF, XB and SX carefully revised this manuscript. XB and SX were responsible for the financial support. All the authors unanimously agreed on the final manuscript.

Data availability

Not applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.05.014.

References

[1] Phelan K, May KM. Mammalian cell tissue culture techniques. Curr Protoc Mol Biol 2017;117:A.3f.1–3f.23.
[2] Borrell B. How accurate are cancer cell lines? Nature 2010;463(7283):858.
[3] Shahbazi A, Abedi Valugerdi M, Kazemi S, Samadi Kochaksaraei S, Naseh MH, Aghdami N, et al. Safety and efficacy of autologous melanocyte/keratinocyte transplantation in patients with refractory stable vitiligo. Dermatology 2023;239(6):919–25.
[4] Jagadeeshaprasad MG, Govindappa PK, Nelson AM, Elfar JC. Isolation, culture, and characterization of primary schwann cells, keratinocytes, and fibroblasts from human foreskin. J Vis Exp 2022;181.
[5] Ścieżyńska A, Nogowska A, Sikorska M, Konys J, Karpińska A, Komorowski M, et al. Isolation and culture of human primary keratinocytes-a methods review. Exp Dermatol 2019;28(2):107–12.
[6] Victoria JG, Wang C, Jones MS, Jaing C, McLoughlin K, Gardner S, et al. Viral nucleic acids in live-attenuated vaccines: detection of minority variants and an adventitious virus. J Virol 2010;84(12):6033–40.
[7] Tekkatte C, Gunasingh GP, Cherian KM, Sankaranarayanan K. "Humanized" stem cell culture techniques: the animal serum controversy. Stem Cell Int 2011;2011:504723.
[8] Cheshire P, Zhafira AS, Banakh I, Rahman MM, Carmichael I, Herson M, et al. Xeno-free expansion of adult keratinocytes for clinical application: the use of human-derived feeder cells and serum. Cell Tissue Res 2019;376(3):389–400.
[9] Aghayan HR, Goodarzi P, Arjmand B. GMP-compliant human adipose tissue-derived mesenchymal stem cells for cellular therapy. Methods Mol Biol 2015;1283:93–107.
[10] Lagerwall C, Shahin H, Abdallah S, Steinvall I, Elmasry M, Sjöberg F, et al. Xeno-free workflow exhibits comparable efficiency and quality of keratinocytes isolated from human skin biopsies. Regen Ther 2021;18:401–7.
[11] Frese L, Darwiche SE, Gunning ME, Hoerstrup SP, von Rechenberg B, Giovanoli P, et al. Optimizing large-scale autologous human keratinocyte sheets for major burns-Toward an animal-free production and a more accessible clinical application. Health Sci Rep 2022;5(1):e449.
[12] Kino-oka M, Taya M. Recent developments in processing systems for cell and tissue cultures toward therapeutic application. J Biosci Bioeng 2009;108(4):267–76.
[13] Doolan BJ, Weaich M, Mamo J, Gupta M. Autologous non-cultured epidermal cellular grafting in the surgical treatment of stable vitiligo: the skin hospital protocol. Dermatology 2022;238(1):167–9.
[14] Michalak-Mićka K, Büchler VL, Zapiórkowska-Blumer N, Biedermann T, Klar AS. Characterization of a melanocyte progenitor population in human interfollicular epidermis. Cell Rep 2022;38(9):110419.
[15] Huang WS, Wang YW, Hung KC, Hsieh PS, Fu KY, Dai LG, et al. High correlation between skin color based on CIELAB color space, epidermal melanocyte ratio, and melanocyte melanin content. PeerJ 2018;6:e4815.
[16] Koster MI, Roop DR. The role of p63 in development and differentiation of the epidermis. J Dermatol Sci 2004;34(1):3–9.
[17] Suzuki D, Senoo M. Increased p63 phosphorylation marks early transition of epidermal stem cells to progenitors. J Invest Dermatol 2012;132(10):2461–4.
[18] Cui YZ, Man XY. Biology of melanocytes in mammals. Front Cell Dev Biol 2023;11:1309557.

[19] Lee A, Lim J, Lim JS. Emerging roles of MITF as a crucial regulator of immunity. Exp Mol Med 2024;56(2):311–8.
[20] Kawakami A, Fisher DE. The master role of microphthalmia-associated transcription factor in melanocyte and melanoma biology. Lab Invest 2017;97(6):649–56.
[21] Hida T, Kamiya T, Kawakami A, Ogino J, Sohma H, Uhara H, et al. Elucidation of melanogenesis cascade for identifying pathophysiology and therapeutic approach of pigmentary disorders and melanoma. Int J Mol Sci 2020;21(17).
[22] Guo H, Xing Y, Liu Y, Luo Y, Deng F, Yang T, et al. Wnt/β-catenin signaling pathway activates melanocyte stem cells in vitro and in vivo. J Dermatol Sci 2016;83(1):45–51.
[23] Solano F. On the metal cofactor in the tyrosinase family. Int J Mol Sci 2018;19(2).
[24] Chen RH, Xiao L, Zhang RZ, Wang SY, Li Y. Dedifferentiation of human epidermal melanocytes in vitro by long-term trypsinization. Cell Tissue Bank 2021;22(1):67–75.
[25] Wang Y, Tissot M, Rolin G, Muret P, Robin S, Berthon JY, et al. Development and validation of a simple method for the extraction of human skin melanocytes. Cytotechnology 2018;70(4):1167–76.
[26] Tuschong L, Soenen SL, Blaese RM, Candotti F, Muul LM. Immune response to fetal calf serum by two adenosine deaminase-deficient patients after T cell gene therapy. Hum Gene Ther 2002;13(13):1605–10.