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A chemically defined culture medium containing Rho kinase inhibitor Y-27632 for the fabrication of stratified squamous epithelial cell grafts

Afag Aslanova [a, b], Ryo Takagi [b], Masayuki Yamato [b], Teruo Okano [b], Masakazu Yamamoto [a, *]

[a] Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan
[b] Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, TWIns, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

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ABSTRACT

With the development of a culture method for stratified squamous epithelial cells, tissue-engineered epithelial cell sheets have been successfully applied as clinical cell grafts. However, the implementation of these cell sheets without the use of any animal-derived materials is highly desirable. In this study, Rho-associated protein kinase inhibitor Y-27632 was used to develop a chemically defined culture medium for the fabrication of stratified epithelial cell grafts consisting of human epidermal and oral keratinocytes, and the proliferation activity, cell morphology, and gene expressions of the keratinocytes were analyzed. The results of a colorimetric assay indicated that Y-27632 significantly promoted the proliferation of the keratinocytes in culture media both with and without fetal bovine serum (FBS), although there were no indications of Y-27632 efficacy on cell morphology and stratification of the keratinocytes in culture medium without any animal-derived materials. The results of quantitative RT-PCR revealed that gene expressions correlated with cell adhesion, cell–cell junction, proliferation markers, and stem/progenitor markers in cultured keratinocytes were not strongly affected by the addition of Y-27632 to the culture medium. Moreover, gene expressions of differentiation markers in stratified keratinocytes cultured in medium without FBS were nearly identical to those of keratinocytes co-cultured with 3T3 feeder cells. Interestingly, the expressions of differentiation markers in cultured stratified keratinocytes were suppressed by FBS, whereas they were reconstructed by either co-culture of a 3T3 feeder layer or addition of Y-27632 into the culture medium containing FBS. These findings indicate that Y-27632 is a useful supplement for the development of a chemically defined culture medium for fabrication of stratified epithelial cell grafts for clinical applications for the purpose of developing the culture medium with a lower risk of pathogen transmission that might arise from animal-derived materials.

1. Introduction

Since the development of a culture method for epidermal keratinocytes by Rheinwald and Green in 1975 [1], clinical applications of cultured epidermal keratinocytes have been used [2–5]. In a similar manner, oral mucosal epithelial cells also have been successfully cultured [6], and tissue-engineered oral mucosal epithelial cell grafts have been used for the treatment of defects [7–12]. Over the years, bovine serum and mouse 3T3 feeder layers have been used as indispensable components for the fabrication of the epithelial cell grafts in which the keratinocytes form stratified epithelium with terminal differentiation and proliferating cells under the differentiated cell layer [1,13–15]. However, prevention of contamination with xenogeneic cells in epithelial cell grafts co-cultured with 3T3 feeder cells is virtually impossible, and therefore, the fabricated epithelial tissue is classified as a xenogeneic product by the US Food and Drug Administration. Bovine serum is the most frequently used supplement for culture media for many types of mammalian cells and is important for the fabrication of cultured epithelial cell grafts. Although these animal-derived materials are useful for fabrication of cell products, the possibility exists for pathogen transmission derived from xenogeneic material. In a previous study, we demonstrated that oral mucosal epithelial

* Corresponding author. Fax: +81 3 3357 5553.
E-mail address: yamamoto.ige@twmu.ac.jp (M. Yamamoto).

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cells cultured on a temperature-responsive cell culture insert with micropores can proliferate and form stratified mucosal tissue without the use of 3T3 feeder cells [16]; moreover, human oral mucosal epithelial cells seeded on the culture insert can also proliferate and form stratified epithelium in culture medium containing autologous human serum, without the use of a mouse feeder layer and bovine serum [17–19]. Hence, the cultured human oral mucosal epithelial cell sheet in this case is classified as an autologous cell product.

However, certain studies have shown that Rho-associated protein kinase inhibitor Y-27632 can increase the number of keratinocytes, thus providing a simple method for preparing large numbers of epithelial cells [20] and dramatically improving the survival of dissociated human embryonic cells [21]. The effect of Y-27632 on indefinite proliferation of keratinocytes in the presence of a mouse feeder layer has been previously shown, and this effect is also reversible once the Rho kinase inhibitor is removed [22]. Palechor-Ceron et al. showed that for Rho kinase inhibition to work in immortalization of human epithelial cells, a serum-containing conditioned medium of irradiated mouse 3T3 feeder layers is also required and that the keratinocyte differentiation is significantly suppressed by addition of Y-27632 [23]. Therefore, the effect of Y-27632 on promotion of proliferation and expansion of the lifespan of cultured keratinocyte is an undeniable phenomenon. However, the effect of Y-27632 on cultured keratinocyte is not well understood under culture conditions without the use of a mouse 3T3 feeder layer and any animal-derived materials, i.e., bovine serum.

On the basis of these previous studies, we hypothesized that Y-27632 is a useful supplement for fabrication of human stratified epithelial cell grafts in culture conditions without any animal-derived material. In this work, the effects of a chemically defined culture medium supplied with Y-27632 on the proliferation, cellular morphology, and gene expressions of human epidermal and oral keratinocytes were analyzed and compared with those from traditional culture methods of human keratinocytes to develop a completely chemically defined culture medium applicable for clinical cases.

2. Materials and methods

2.1. Culture medium

Low [Ca^{2+}] medium (LCM) was used for subculture of human epidermal and oral keratinocytes. Keratinocyte culture medium (KCM) was used to induce differentiation of serially cultured human keratinocytes. The LCM consisted of Serum Free Medium for Human Keratinocyte (DS Pharma Biomedical, Osaka, Japan) supplemented with 2% bovine pituitary extract (BPE; DS Pharma Biomedical), 0.4 μg/mL hydrocortisone (Wako Pure Chemical Industries, Osaka, Japan), 2.0 nM triiodothyronine (MP Biomedicals, CA, USA), 1.0 nM cholera toxin (Calbiochem, Merck Millipore, Darmstadt, Germany), 5.0 μg/mL insulin (Gibco, Life Technologies, CA, USA), 5.0 μg/mL transferrin (Gibco, Life Technologies), and 10 ng/mL EGF (Protein Express, Chiba, Japan). The KCM consisted of mixture of three parts Dulbecco's modified Eagle's medium (Sigma–Aldrich, MO, USA) and one part nutrient mixture F-12 Ham (Sigma–Aldrich) with the same supplements as LCM except BPE. Fetal bovine serum (FBS) and Y-27632 (Wako Pure Chemical Industries) were added to the KCM, depending on the culture conditions.

2.2. Culture of human epidermal keratinocytes

Normal human epidermal keratinocytes (Lot No. 0000173635 and 0000295586, Lonza, Basel, Swiss) and human oral keratinocytes (Lot No. 5973, ScienCell Research Laboratories, CA, USA) were serially cultured in LCM and cryopreserved at passage 2. The cryopreserved keratinocytes were cultured in LCM for approximately 1 week and then treated with 0.25% (w/v) trypsin and 0.1% (w/v) EDTA (Sigma–Aldrich) for 3 min at 37 °C. The cell suspension was used for the proliferation assay, fabrication of cultured stratified epithelial cell sheet, and gene expression analysis.

2.3. Cell proliferation assay

A colorimetric method using crystal violet was performed as a cell-proliferation assay [24]. Subcultured keratinocytes were seeded on 48-well culture plates at a density of 1×10^4 cells/cm^2 and cultured for 3 days. After cultivation, the cells were washed with PBS and fixed with methanol (Wako Pure Chemical Industries) containing 0.4% (w/v) crystal violet (Sigma–Aldrich) for 10 min at room temperature. The fixed cells were thoroughly washed with pure water, and the absorbed crystal violet with the cells was eluted in pure water containing 30% (v/v) ethanol (Wako Pure Chemical Industries) and 1% (v/v) acetic acid for 10 min at room temperature. The concentration of the eluted crystal violet was measured by a SpectraMax M2e instrument (Molecular Devices, CA, USA).

2.4. Fabrication of cultured human keratinocyte sheet

Subcultured keratinocytes were seeded onto a temperature-responsive cell culture insert (CellSeed, Tokyo, Japan) in KCM at a density of 1–4 x 10^4 cells/cm^2 and cultured at 37 °C. The culture media were replaced every 2 or 3 days. After the cells became confluent on the culture surface, the cells were cultured for another 5 days with replacement of culture medium performed every 1 or 2 days. The cultured keratinocytes were cultured at 20 °C for 30 min and were harvested as cell sheets for histological analysis. The fabricated human keratinocyte sheets were fixed with 10% formalin solution (Wako Pure Chemical Industries) and processed into a 3-μm-thick paraffin-wax-embedded section to confirm stratification of the cell sheets. Hematoxylin and eosin (HE) staining was performed via conventional methods. Observation of the slides was performed with a Nikon Eclipse E800 Microscope and NIS-Elements (Nikon, Tokyo, Japan).

2.5. Gene expression assay

Subcultured human epidermal keratinocytes were seeded onto 6-well plates at a density of 1×10^4 cells/cm^2 and cultured for 2 days at 37 °C. The keratinocytes were observed by phase-contrast microscope to confirm that the cells were not confluent, and the total RNAs of the keratinocytes were prepared with RNeasy Plus Mini Kit (QUIAGEN, Hilden, Germany) according to the manufacturer's protocol accompanying the reagents. The total RNAs were used as samples of proliferating keratinocytes for gene expression analysis. Subcultured human keratinocytes were also seeded on 6-well cell culture inserts at a density of 1–4 x 10^4 cells/cm^2 and cultured at 37 °C. After the cells became confluent on the culture insert, the cells were cultured for another 3 days with replacement of culture medium performed every 1 or 2 days, and the total RNAs of the keratinocytes were prepared using an RNeasy Plus Mini Kit (QUIAGEN). The total RNAs were used as samples of stratified keratinocytes for gene expression analysis. Reverse transcription reactions were performed with First-strand cDNA Synthesis (OriGene, MD, USA) according to the manufacturer's protocol accompanying the reagents. A gene expression assay using a 6-FAM dye-labeled TaqMan probe (Applied Biosystems, Life Technologies, CA, USA) was performed by Biomark HD on a 48.48 Dynamic Array instrument (Fluidigm, CA, USA) following the manufacturer's instructions.

3. Results

3.1. Proliferation assay

A colorimetric proliferation assay was performed to confirm the effect of the ROCK inhibitor Y-27632 contained in the KCM either with or without 5%FBS on proliferation of keratinocytes. Cultured human oral keratinocytes were stained with crystal violet solution (Fig. 1A). The crystal violet was eluted from the cells using 30% ethanol and 1% acetic acid; thereafter, the amount of crystal violet absorbed by the keratinocytes was measured in each solution (Fig. 1B). The correlation coefficient between the seeding density of keratinocytes and the amount of crystal violet absorbed by the cultured keratinocytes was 0.997 (data not shown). The Y-27632 significantly promoted the proliferation of oral keratinocytes in culture media both with and without FBS ($p < 0.01$). The same assay was performed for human epidermal keratinocytes, and similar to the oral keratinocytes, the proliferation was significantly promoted by Y-27632 (data not shown). These results indicated that Y-27632 significantly promoted cell proliferation of cultured human keratinocytes in culture medium without any animal-derived materials.

3.2. Morphology of the cultured keratinocytes

The efficacies of each culture medium on the morphology of epidermal keratinocytes were measured on day 2 after seeding in the culture dish (Fig. 2A). The cellular morphology of the keratinocytes cultured in 5%FBS-KCM was highly altered by addition of Y-27632, although there was no indication of efficacy of Y-27632 on the cell morphology of keratinocytes cultured in FBS(−)KCM. The keratinocytes seeded on culture inserts were cultured for another 3 days after cells became confluent for fabrication of stratified epithelium; the morphology is shown in Fig. 2B. Comparison of the keratinocytes cultured in KCM in the presence of 5%FBS with or without Y-27632, showed that the keratinocytes cultured in 5%FBS-KCM with Y-27632 had higher cell density than the cells cultured without the addition of Y-27632. Human epidermal keratinocytes were also cultured on temperature-responsive cell culture inserts, and to confirm the stratification, the cells were harvested as cultured epithelial sheets by reducing the temperature from 37 °C to 20 °C at day 5 after cells became confluent (Fig. 3). In all culture conditions, the keratinocytes successfully formed stratified epithelium with the presence of stratum granulosum with possible keratinhyalin granules, except those prepared in 5%FBS-KCM medium without Y-27632.

3.3. Gene expression assay

Human epidermal keratinocytes were cultured to compare the effect of each culture medium on gene expression, and the results of the gene expression assay are presented in the heat map shown in Fig. 4. The results indicated that human keratinocytes cultured in LCM expressed mesenchymal cell markers, such as vimentin (*VIM*) and fibronectin (*FN1*) [25], and the expressions were significantly suppressed in stratified keratinocytes cultured on cell culture inserts. Similarly to those gene expressions of *VIM* and *FN1*, stem/progenitor and proliferation markers, such as tumor protein p63 (*TP63*), Ki-67 (*MKI67*), and BMI1 polycomb ring finger oncogene

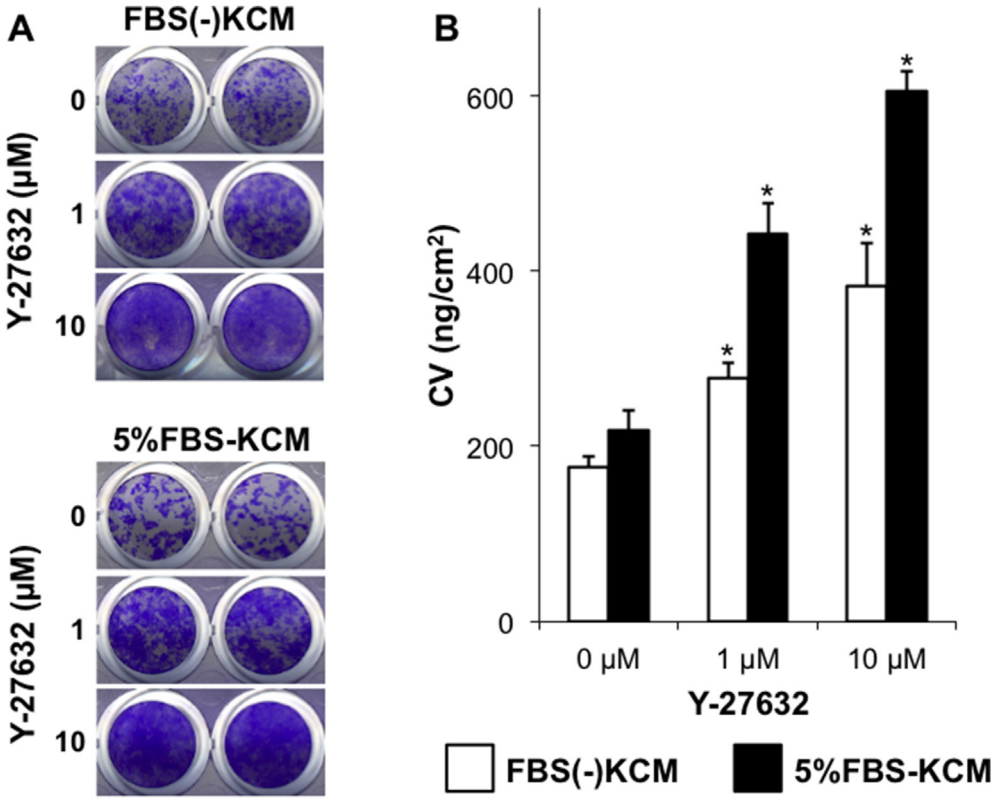

Fig. 1. The effect of Y-27632 on the proliferation of human keratinocytes under chemically defined culture conditions. (A) Human oral keratinocytes were cultured on 48-well plates in keratinocyte culture medium (KCM) with or without 5% fetal bovine serum (FBS) supplied with 0, 1, and 10 μM Y-27632. After cultivation for 3 days, the keratinocytes were stained with crystal violet (CV). FBS(−)KCM showing serum-free culture condition. (B) The CV absorbed by the cultured keratinocytes was eluted by pure water containing 30% ethanol and 1% acetic acid, and the concentration was measured. Amounts of absorbed CV are shown as weight per culture area of the 48-well plates (ng/cm^2). The means were compared using Dunnett's test, in which the means of FBS(−)KCM and 5%FBS-KCM without Y-27632 were used as control groups. *: $p < 0.01$.

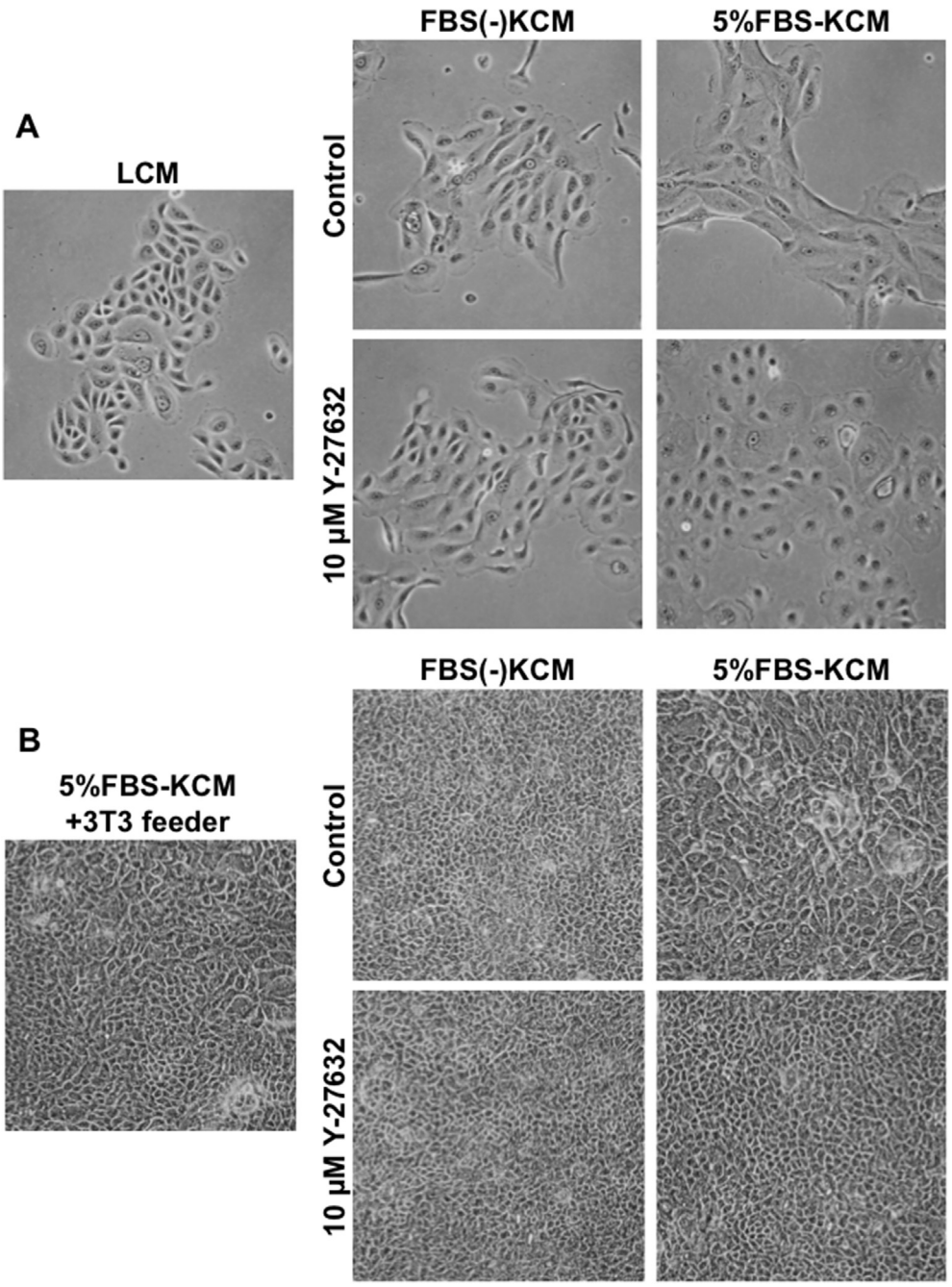

Fig. 2. Observation of cultured human epidermal keratinocytes in different culture conditions using Y-27632. (A) Subcultured human epidermal keratinocytes were seeded in low [Ca^{2+}] medium (LCM) and keratinocyte culture medium (KCM) with or without 5% fetal bovine serum (FBS). The Y-27632 was added to KCM containing FBS (5%FBS-KCM) and serum-free KCM (FBS(−)KCM). Control KCMs were not treated with Y-27632. Observation was performed after 2 days of the cultivation. (B) Subcultured human epidermal keratinocytes were seeded on a cell culture insert to induce stratification and cultured in KCM with or without 5%FBS. The Y-27632 was added to 5%FBS-KCM and FBS(−)KCM. Control KCMs were not treated with Y-27632. To compare the cell morphology of keratinocytes cultured by the traditional stratification method, NIH-3T3 cells treated with mitomycin C were seeded on 6-well plates, and keratinocytes were seeded on cell culture inserts placed on the 6-well plates and cultured in 5%FBS-KCM. After the keratinocytes became confluent, the cells were cultured for another 3 days and then observed.

(*BMI1*), were also suppressed in stratified keratinocytes compared with proliferating keratinocytes that had not yet become confluent. Gene expressions of keratin 1 (*KRT1*), keratin 10 (*KRT10*), filaggrin (*FLG*), loricrin (*LOR*), and transglutaminase 1 (*TGM1*), which are known as differentiation markers of keratinocytes, were not induced in proliferating keratinocytes; however, in stratified keratinocytes, the expression of these markers in 5%FBS-KCM was significantly lower than in other culture conditions. Interestingly, the suppression of gene expressions of differentiation markers by FBS was recovered by the addition of Y-27632, similar to that observed with the 3T3 feeder layer. The differences in gene expressions correlated with cell adhesion, cell–cell junction, proliferation marker, and stem/progenitor marker were not distinguishable in each culture medium.

4. Discussion

Previous reports show that primary epidermal keratinocytes derived from human tissue specimen promote proliferation and clonogenic ability with the addition of Y-27632 into the culture

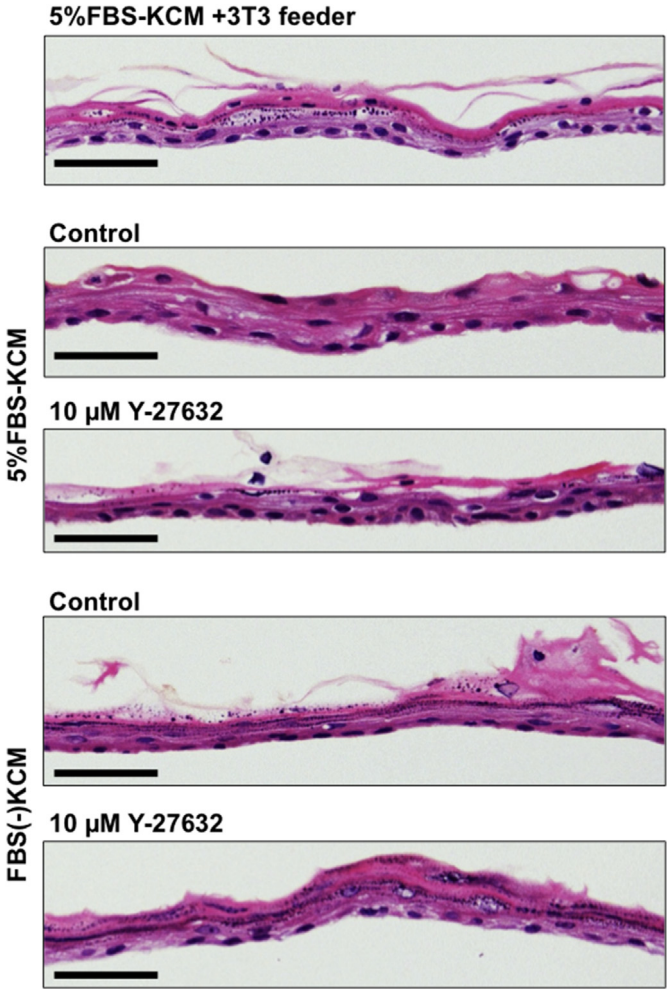

Fig. 3. The effect of Y-27632 on stratification of keratinocytes. Subcultured human epidermal keratinocytes were seeded on a temperature-responsive cell culture insert to induce stratification and cultured in keratinocyte culture medium (KCM) with or without 5% fetal bovine serum (FBS). The Y-27632 was added to KCM containing FBS (5%FBS-KCM) and serum-free KCM (FBS(−)KCM). Control KCMs were not treated with Y-27632. To compare the cell morphology of keratinocytes cultured with the traditional stratification method, NIH-3T3 cells treated with mitomycin C were seeded on 6-well plates, and keratinocytes were seeded on temperature-responsive cell culture inserts placed on the 6-well plates and cultured in 5%FBS-KCM. After the keratinocytes became confluent, the cells were cultured for another 5 days and harvested from the culture surface by reducing the temperature from 37 °C to 20 °C for 30 min. Bars: 50 μm.

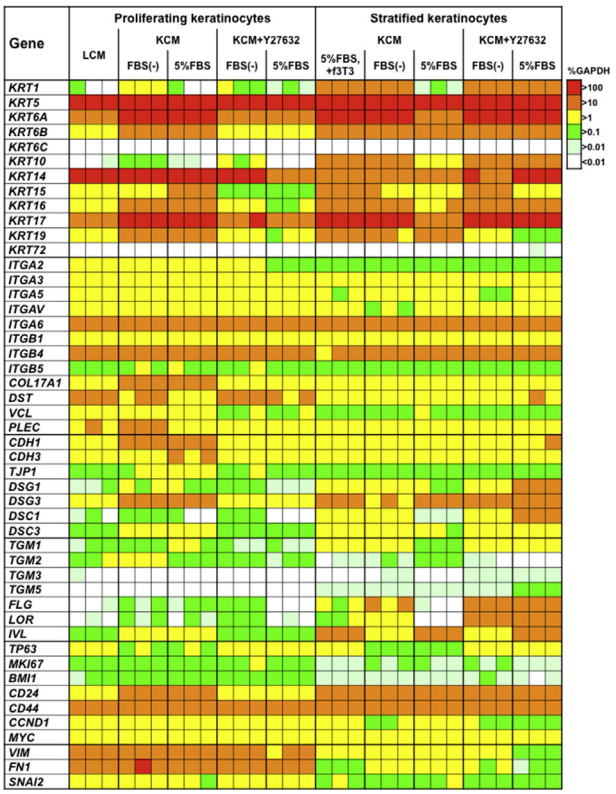

Fig. 4. The effect of Y-27632 on gene expressions of cultured human epidermal keratinocytes. Subcultured human epidermal keratinocytes were seeded in low [Ca^{2+}] medium (LCM) and keratinocyte culture medium (KCM) with or without 5% fetal bovine serum (FBS). The Y-27632 was added to KCM containing FBS (5%FBS) and serum-free KCM (FBS(−)). Control KCMs were not treated with Y-27632. Total RNA was prepared after 2 days of cultivation. These samples are shown as proliferating keratinocytes. Subcultured human epidermal keratinocytes were also seeded on a cell culture insert to induce stratification and cultured in KCM with or without 5%FBS. The Y-27632 was added to KCM containing FBS (5%FBS) and serum-free KCM (FBS(−)). Control KCMs were not treated with Y-27632. To confirm gene expressions of keratinocytes cultured by the traditional stratification method, NIH-3T3 cells treated with mitomycin C (f3T3) were seeded on 6-well plates, and keratinocytes were seeded on cell culture inserts placed on the 6-well plates and cultured in 5%FBS-KCM. After the keratinocytes became confluent, the cells were cultured for another 3 days and total RNA was prepared. These samples are shown as stratified keratinocytes. The results of gene expressions correlated with differentiation, cell adhesion, cell–cell junction, stem/progenitor marker, and mesenchymal cell marker are shown in the heat map and indicated in *%GAPDH*.

medium [20]. Cultured human keratinocytes are efficiently immortalized by Y-27632 [26], whereas Y-27632 cooperates with diffusible factors released by lethally irradiated 3T3 feeder cells and induces conditional immortalization of cultured human keratinocyte [23]. These reports were demonstrated under culture conditions with bovine serum and mouse 3T3 feeder layer, whereas in the present study, the results of proliferation assays of human keratinocytes are demonstrated with neither serum nor feeder layer. Proliferation of human keratinocytes was significantly promoted by the addition of Y-27632 into KCM. Interestingly, the addition of FBS into KCM supplied with Y-27632 significantly promoted the proliferation of the keratinocytes, although no significant difference was observed in the proliferation between FBS(−)KCM and 5%FBS-KCM. Further experiments are required to better understand the mechanism of synergistic promotion of keratinocyte proliferation by serum and Y-27632, and the clarification of the mechanism will provide important knowledge for development of chemically defined culture medium for effective fabrication of stratified epithelial cell sheets for future clinical applications.

Suppression of differentiation of keratinocytes is one of the primary reasons for inducing the proliferation of keratinocyte using culture medium supplied with Y-27632. Terminal differentiation of epidermal keratinocytes is induced by cultivation in single-cell suspension, and the differentiation is suppressed by Rho inhibitor C3 ADP-ribosyltransferase and Y-27632 [27]. The activation of Notch1 by treatment with EDTA induces the activation of Rho kinase, differentiation of keratinocytes, and reduction of the clonogenicity, whereas the inhibition of ROCK1 suppresses the differentiation and reduction of the clonogenicity [28]. However, epidermal keratinocytes cultured on de-epidermized dermis form stratified epidermal tissue with expressions of differentiation markers of keratinocyte, and the expression is not affected by addition of Y-27632 in the culture medium [29]. In the case of the

cornea, limbal epithelial cells cultured on a cell culture insert facilitate long-term maintenance of the stem/progenitor cells with the addition of Y-27632, and the cultured cell sheet forms stratification with normal differentiation similar to that of corneal epithelium [30]. Interestingly, skin epidermis of mice with epidermal keratinocytes with specific deletion of RhoA show normal development with differentiation despite a reduction of the phosphorylation of the myosin light chain and cofilin [31]. In the present study, expressions of genes correlated with terminal differentiation in stratified keratinocytes cultured on cell culture insert were not suppressed by Y-27632 in culture medium without FBS; additionally, the expressions were nearly identical to those of stratified keratinocytes co-cultured with 3T3 feeder cells. Therefore, the effect of Rho kinase on the differentiation of keratinocytes depends on the environment of the keratinocyte, and it is possible that the differentiation is not affected by Y-27632 *in vivo* and when culture conditions induce the stratification of the epithelial cell.

The cellular morphology of proliferating keratinocytes was highly alternated by the addition of Y-27632 under culture condition supplemented with FBS, although there was no indication of the efficacy of Y-27632 on the cell morphology of the keratinocytes cultured in FBS(−)KCM (Fig. 2A). Additionally, gene expressions correlated with differentiation of keratinocytes were also significantly alternated by the addition of Y-27632 into 5%FBS-KCM. Although details of the mechanism are not fully elucidated in the present report, the identification of the soluble factor(s) in FBS may be useful for modulation of the differentiation of keratinocytes cultured in chemically defined culture conditions for clinical application and basic research.

The aim of our study was to develop a method for fabricating stratified squamous epithelial cell sheets that does not entail the use of animal-derived materials for use in future clinical studies, thereby reducing possible infectious transmission to the application site. In this study, we used lots of keratinocytes derived from different donors to confirm the reproducibility of the experiment. Although the efficacy of Y-27632 on long-term serial cultivation was not confirmed in this study, serially cultured keratinocytes in LCM expressed differentiation markers under the varying culture conditions, including FBS-depleted medium in the presence of Y-27632 in which cells could proliferate and stratify. Moreover, the differences in gene expressions correlated with cell adhesion, cell–cell junction, proliferation markers, and stem/progenitor markers in stratified keratinocytes fabricated in FBS(−)KCM supplied with Y-27632 were not distinguishable from those of the keratinocytes co-cultured with 3T3 feeder cells in 5%FBS-KCM. Therefore, human keratinocytes subcultured in conventional low-calcium medium without use of FBS and feeder cells is a promising cell source for the fabrication of cultured epithelial cell grafts under chemically defined culture conditions. In this culture method, because autologous human serum is not used to fabricate the cultured human epithelial cell grafts, the cell graft may be useful as allogeneic graft for the treatment of severe burns and chronic skin ulcers.

Conflict of interest

The authors declare no conflict of interest.

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Transparency document

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