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Original Article

Laminin-511-derived recombinant fragment and Rho kinase inhibitor Y-27632 facilitate serial cultivation of keratinocytes differentiated from human embryonic stem cells

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ABSTRACT

Introduction: Keratinocytes derived from pluripotent stem cells have a short proliferative lifespan under conventional culture conditions that are optimized for keratinocytes. Recently, a Rho kinase inhibitor, Y-27632, had been used as a standard supplement for culture medium in which the proliferative lifespan of postnatal keratinocytes was markedly expanded. In addition, recombinant human laminin-511 was demonstrated to be an adhesive ligand for promoting proliferation of cultured epidermal keratinocytes. Based on this knowledge, efficacies of Y-27632 and a laminin511-derived recombinant fragment, known as laminin-511 E8 fragment (LN-511-E8), were evaluated for establishing cultivation methods of keratinocyte differentiated from human embryonic stem cells (hESC).

Methods: Differentiated cells from hESCs, which were established with clinical grade in previous study, were seeded onto culture dishes coated with LN-511-E8 and co-cultured with a mouse feeder layer in serum-free medium supplemented with Y-27632. Before serial cultivation, hESC-derived keratinocytes were separated from other differentiated cells by trypsinization. The isolated hESC-derived keratinocytes were used for evaluating clonogenicity, gene expression analysis for keratinocyte markers, potency of terminal differentiation by air-lifting culture, and long-term proliferation activity by serial cultivation. Moreover, efficacies of Y-27632, LN-511-E8, and mouse feeder layer were evaluated on proliferation of hESC-derived keratinocytes.

Results: hESC-derived keratinocytes with activity of clonal growth were successfully isolated by trypsinization and exhibited potency of differentiation to form stratified epidermal equivalents with expressions of progenitor and differentiation markers of epidermal keratinocyte. Y-27632 and LN-511-E8 were required for maintaining the proliferative activity of the hESC-derived keratinocytes in serially cultivation using mouse feeder layer with stable doubling time during logarithmic growth phase.

Conclusions: These results indicate the utility of Y-27632 and LN-511-E8 for serial cultivation of hESC-derived keratinocytes, which have a potential for fabricating allogeneic cellular products in clinical situations for regeneration of stratified epithelial tissues.

1. Introduction

Human epidermal keratinocytes were the world's first somatic cells for fabrication of transplantable cellular grafts as clinical products [1,2]. The keratinocytes are prepared by a culture system known as the feeder layer method and are applied as cellular grafts for treatment of large burns, giant congenital nevi, and skin ulcers

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[3–6]. Moreover, cultured keratinocytes are also used for gene therapy of junctional epidermolysis bullosa [7,8]. Because there is no limitation on cell replication in pluripotent stem cells, such as human embryonic stem cell (hESC), stem cells are also useful candidate as cell sources for preparing cultured keratinocyte grafts for skin defects and diseases [9–11]. Indeed, there are many reports demonstrating culture methods for differentiation of hESCs into keratinocytes [12].

hESCs spontaneously differentiate into keratinocytes in *in vivo* and *in vitro* environments as determine by the keratinocyte markers: tumor protein p63 (TP63), keratin 14 (KRT14), basonuclin, and involucrin (IVL), where are expressed during differentiation [13]. However, the cellular properties of keratinocytes differentiated from hESCs differ markedly from postnatal keratinocytes. Although a conventional feeder layer method is well-optimized for maintaining the proliferative activity of postnatal keratinocytes, the lifespan of hESC-derived keratinocytes is extremely limited (<15 population doublings), and transduction with the E6E7 gene of HPV16 is required for clonal isolation and expansion of hESC-derived keratinocytes [14]. As a consequence, development of a fabrication method for keratinocyte grafts from hESC is hampered by the low replicative potential.

Recently, a Rho kinase inhibitor and laminin-511 have been employed as standard supplements in cultivation of primary keratinocytes. Y-27632, a Rho kinase inhibitor, affects differentiation, clonogenicity, and proliferation of keratinocytes *in vitro* [15–17]. Indeed, culture medium supplemented with Y-27632 allows human epidermal keratinocytes to proliferate and form transplantable stratified epithelium without a mouse feeder layer and fetal bovine serum (FBS) [18]. Laminin-511 is a ubiquitous laminin isoform and a component of the subepidermal basal membrane with other laminin isoforms [19]. In order to establish a chemically defined culture condition for expansion of human epidermal keratinocytes to treat skin defects, recombinant human laminin-511 is a potential reagent for replacing the murine feeder layer [20]. Human laminin-511-derived recombinant fragment, known as laminin-511 E8 fragment (LN-511-E8), is used in xeno-free culture condition for hESCs and human induced pluripotent stem cells (hiPSCs) and supports efficient adhesion and expansion of the stem cells [21]. The molecular structure of LN-511-E8 is heterotrimer of C-terminal regions of α5, β1, and γ1 chains of laminins in which active binding site of integrin α6β1 is retained [22–24]. Interestingly, LN-511-E8 is used for fabrication of transplantable human corneal epithelial cell sheets from hiPSCs [25]. Based on the results of the previous studies, we hypothesized that Y-27632 and LN-511-E8 would be effective for cultivation of hESC-derived keratinocytes. In the present study, keratinocytes spontaneously differentiated from hESCs were separated by sensitivity to trypsin and ethylenediaminetetraacetic acid (EDTA) for enrichment of the keratinocyte population. Clonogenicity, gene expressions of keratinocyte markers, and potency of terminal differentiation of the isolated hESC-derived keratinocytes were evaluated by colony formation assay, qRT-PCR, and immunohistological analysis of epidermal equivalents prepared by air-lifting cultures. Moreover, hESC-derived keratinocyte was used for confirming proliferative activity in long-term serial cultivation using mouse feeder layer with LN-511-E8 and Y-27632.

2. Materials and methods

2.1. Ethical statement

All experiments with human cells and tissue were approved by the Institutional Review Board at the National Institute of Biomedical Innovation. Informed consent was obtained from the parent of the patient. The derivation and cultivation of ESC lines were performed in full compliance with “the Guidelines for Derivation and Distribution of Human Embryonic Stem Cells (Notification of the Ministry of Education, Culture, Sports, Science, and Technology in Japan (MEXT), No. 156 of August 21, 2009; Notification of MEXT, No. 86 of May 20, 2010)” and “the Guidelines for Utilization of Human Embryonic Stem Cells (Notification of MEXT, No. 157 of August 21, 2009; Notification of MEXT, No. 87 of May 20, 2010)”.

2.2. Culture and differentiation of hESCs

SEES-2 which was a hESC line established in our previous study [26] were routinely cultured in ESC culture medium with feeder layer derived from mouse embryonic fibroblasts. The fibroblasts were isolated from ICR mouse fetuses at 12.5 d gestation, serially cultured 2 times, and gamma-irradiated at 30 Gy (Hitachi, MBR-1520 R-3) for preparing the feeder layer. The ESC media consisted of KnockoutTM-Dulbecco's modified Eagle's medium (KO-DMEM; Thermo Fisher Scientific, MA, USA) supplemented with 20% KnockoutTM-Serum Replacement (KO-SR; Thermo Fisher Scientific), 2 mmol/L Glutamax-I (Thermo Fisher Scientific), 0.1 mmol/L non-essential amino acids (NEAA; Thermo Fisher Scientific), 1 mmol/L sodium pyruvate (Thermo Fisher Scientific), and 50 ng/mL recombinant human full-length bFGF (Thermo Fisher Scientific).

To generate embryoid bodies (EBs), 5×10^3 cells/well ESCs were dissociated into single cells with Accutase (Thermo Fisher Scientific) after exposure to Y-27632 (Fujifilm Wako Pure Chemicals, Osaka, Japan), and cultivated in the 96-well plates in the EB medium consisting of KO-DMEM supplemented with 20% KO-SR, 2 mmol/L GlutaMAX-I, 0.1 mmol/L NEAA, and 1 mmol/L sodium pyruvate for 4 days. The EBs were transferred to the 24-well plates coated with collagen type I, and cultivated in the XF32 medium consisting of KO-DMEM supplemented with 15% Knockout Serum Replacement XF CTS (Thermo Fisher Scientific), 2 mmol/L GlutaMAX-I, 0.1 mmol/L NEAA, 1 mmol/L sodium pyruvate, 50 μg/mL l-ascorbic acid 2-phosphate, 10 ng/mL heregulin-1β (R&D Systems, MN, USA), 200 ng/mL recombinant human IGF-1 (Sigma–Aldrich), and 20 ng/mL human bFGF for 35 days. The differentiated cells were further co-cultured with feeder layer of the mouse embryonic fibroblasts and propagated in ESTEM-HE medium (GlycoTechnica, Kanagawa, Japan) containing Wnt3a and R-spondin 1 at 37 °C in a humidified 5% CO_2 atmosphere [27]. When the cultures became subconfluence, the cells were harvested with 0.25% trypsin and 1 mmol/L EDTA (Fujifilm Wako Pure Chemicals) and re-plated at a density of 1/4 confluent on 100 mm culture dishes. Medium changes were carried out every three days thereafter. The differentiated hESCs were serially cultured and cryopreserved at passage 4.

2.3. Purification and serial cultivation of hESC-derived keratinocytes

Mouse fibroblasts derived from bone marrow of calvarium were serially cultured in alpha modification of Eagle's minimum essential medium (α-MEM; Thermo Fisher Scientific) containing 10% FBS (Thermo Fisher Scientific), 100 unit/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific), and 0.25 μg/mL amphotericin B (Bristol-Myers Squibb, NY, USA) for preparing feeder layers. Preparation method of the feeder layers was implemented in accordance with previous study [28]. Briefly, the mouse fibroblasts treated with 10 μg/mL mitomycin C (nacalai tesque, Kyoto, Japan) for 2 h were seeded at 2.5×10^4 cells/cm^2 onto cell culture dishes coated with LN-511-E8 (Nippi, Tokyo, Japan) according to manufacture's protocols.

The cryopreserved differentiated hESCs were seeded at a density of 2.9 × 10^4 cells/cm^2 onto culture dishes coated with LN-511-E8, and co-cultured with the feeder layer in Defined Keratinocyte Serum-free Medium (DK-SFM; Thermo Fisher Scientific) supplemented with 10 μmol/mL Y-27632, 100 unit/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B at 37 °C in a humidified 5% CO_2 atmosphere. For separating hESC-derived keratinocytes to enrich the cell population, additional trypsinizations were implemented before serial cultivation at passages 5, 6, and 7 (Fig. 1A). The differentiated hESCs were treated with trypsin–EDTA at 37 °C in a humidified 5% CO_2 atmosphere for 3–6 min, and supernatants of this treatment were discarded after the incubation for removing cells not exhibiting keratinocyte-like cellular morphology. After this 1st trypsinization, the cells adhering on the culture dishes were also treated with fresh trypsin–EDTA for serial cultivation. For confirming proliferative non-keratinocyte cells in the differentiated hESCs at passage 6, cell suspension of 1st trypsinization were also cultured in serial culture condition and conventional culture condition for fibroblast by using normal culture dish and α-MEM containing 10% FBS, penicillin, and streptomycin. After passage 8, the hESC-derived keratinocytes were serially cultured without the additional trypsinization until the proliferation rate decreased. At passage 13, the hESC-derived keratinocytes were also cultured in α-MEM containing 10% FBS for confirming the non-keratinocyte cells. Population doubling levels (PDL) and doubling times of the hESC-derived keratinocytes were calculated during serial cultivation. For confirming effect of trypsinization on separation, the cells differentiated from SEES-2 were also serially

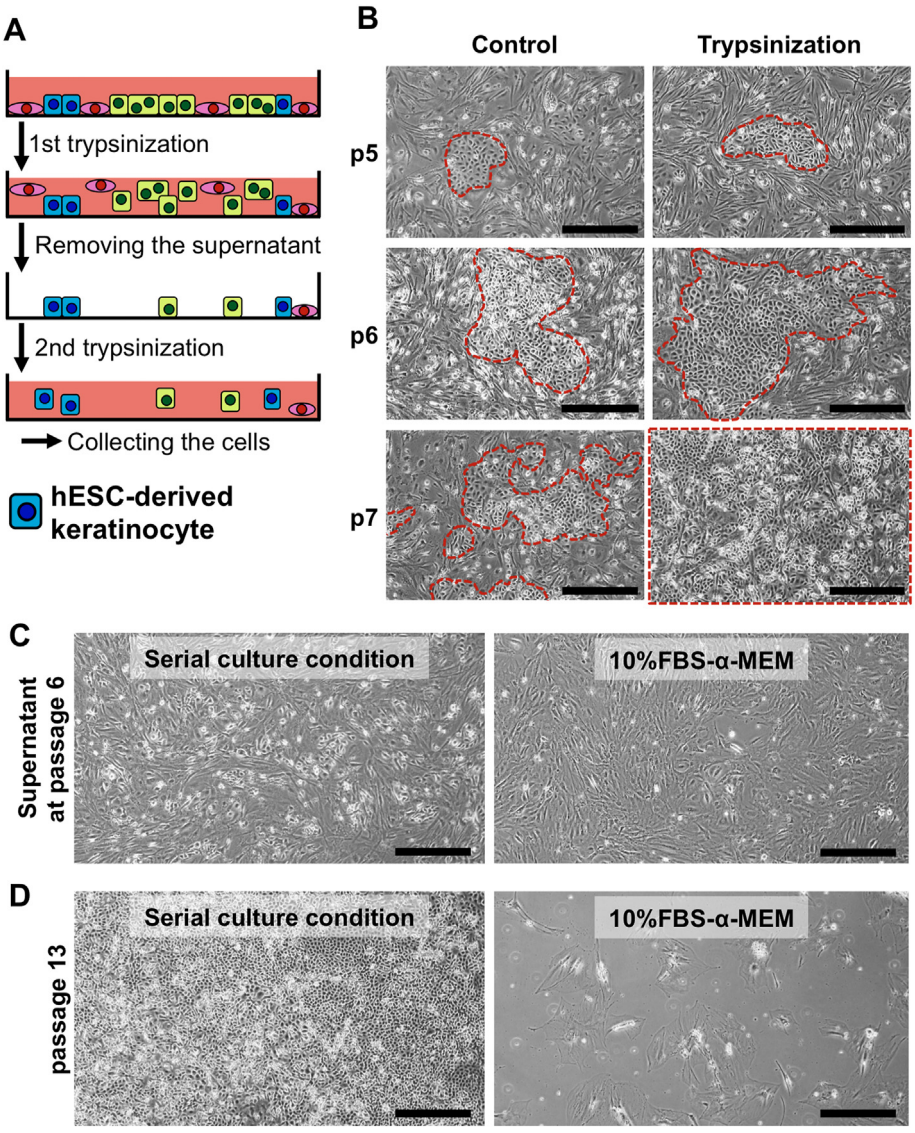

Fig. 1. Separation and enrichment of human embryonic stem cell (hESC)-derived keratinocytes by trypsinization and serial cultivation. (A) Schematic illustration of how to use trypsinization for separating hESC-derived keratinocytes from differentiated hESCs. Before serial cultivation, cells differentiated from hESCs were treated with trypsin–EDTA, and supernatant was discarded to remove cells without keratinocyte-like morphology. After trypsinization, cells adhering to the culture dishes were harvested for serial cultivation. (B) Cellular morphology of hESC-derived keratinocytes at passage 5, 6, and 7 in the control and trypsinization groups. Left column: control group and right column: trypsinization group. Upper row: passage 5 (p5), middle row: passage 6 (p6), and bottom row: passage 7 (p7). The keratinocytes in the differentiated hESCs are shown by dashed red lines. Scale bars indicate 500 μm. (C) Morphology of cells collected by 1st trypsinization at passage 6 (PDL2.0). The cells were cultured in serial culture condition (left). The cells were also seeded onto normal culture dishes without feeder layer and cultured in α-MEM supplied with 10% fetal bovine serum (FBS), in which fibroblasts showed proliferative activity (10%FBS-α-MEM, right). Under this culture condition, non-keratinocyte cells were observed in 10%FBS-α-MEM. Scale bars indicate 500 μm. (D) Morphologies of hESC-derived keratinocyte at passage 13 (PDL23) in the serial cultivation (left) and fibroblast culture condition (right). The keratinocytes were also seeded onto normal culture dishes without feeder layer and cultured in 10% FBS-α-MEM. Under this culture conditions, proliferative non-keratinocyte cells were not observed in hESC-derived keratinocytes. Scale bars indicate 500 μm.

cultured without the trypsinization before serial cultivation, and these cells were used as a control group.

2.4. Clonogenicity analysis for hESC-derived keratinocytes

Keratinocytes derived from hESCs in the control and trypsinization groups at passage 7 (PDL3.7) were seeded at a density of 100 cells/well onto 6-well plates, and co-cultured with the mouse feeder layer in keratinocyte culture medium (KCM) supplemented with 10 μmol/L Y-27632. The composition of KCM is described in a previous report [29]. Briefly, KCM consists of mixture of three parts of Dulbecco's modified Eagle's medium (Sigma–Aldrich, MO, USA) and one part of Ham's F-12 nutrient mixture (Thermo Fisher Scientific) supplemented with 100 unit/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, 5% FBS, 5 μg/mL insulin (Humulin; Eli Lilly, IN, USA), 10 ng/mL human recombinant epidermal growth factor (Higeta Shoyu, Chiba, Japan), 1 nmol/L cholera toxin (Fujifilm Wako Pure Chemicals), 2 nmol/L triiodothyronine (Fujifilm Wako Pure Chemicals), and 0.4 μg/mL hydrocortisone (Saxizon; Teva Takeda Pharma, Aichi, Japan). After approximately 2 weeks of cultivation, the hESC-derived keratinocytes were stained with crystal violet, and percentages of colony formation efficiencies were calculated by a conventional method [3]. Colonies of hESC-derived keratinocytes were classified into three groups: keratinocyte colonies, non-stratified epithelial colonies, and fibroblast-like colonies.

2.5. Gene expression analysis of hESC-derived keratinocytes

Gene expression analysis for keratin 14 (*KRT14*), tumor protein p63 (*TP63*), involucrin (*IVL*), and filaggrin (*FLG*) were carried out in accordance with previous our study with sequence data of the primers [30]. In brief, total RNA samples were prepared from hESC-derived keratinocytes at passage 5 and 9 by RNeasy Plus kit (Qiagen, Hilden, German) for cDNA synthesis by SuperScript III Reverse Transcriptase (Thermo Fisher Scientific). The mRNA expressions were analyzed by Platinum SYBR Green qPCR SuperMix-UDG (Thermo Fisher Scientific) and Applied Biosystems Quantstudio 12K Flex Real-Time PCR System (Thermo Fisher Scientific). The expression levels were normalized by expressions of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and shown as means of percent of *GAPDH* (*%GAPDH*) with standard deviations. Total RNA samples were prepared from human postnatal epidermal keratinocytes at passage 3 and used as positive controls for detecting the gene expressions. The keratinocyte were co-cultured with mouse feeder layer in KCM supplemented with 10 μmol/L Y-27632 for 8 days. After the cultivation, the keratinocytes were cultured in KCM without Y-27632 for 3 days to induce terminal differentiation.

2.6. Air-lifting culture method

Keratinocytes derived from hESCs at passage 16 (PDL31) were used for fabrication of stratified epidermal equivalents by air-lifting culture according to a conventional method (Fig. 4A). Human postnatal epidermal keratinocytes at passage 5 were used as control cells. In brief, mouse feeder layers were seeded at a density of 2.5×10^4 cells/cm^2 onto 6 well plates and cell cultured insert (Corning, NY, USA) coated with LN-511-E8. These keratinocytes derived from hESCs and postnatal epidermis were seeded onto the inserts at a density of 1×10^4 cells/cm^2 and 0.5×10^4 cells/cm^2, respectively. These keratinocytes were cultured in KCM supplemented with 10 μmol/L Y-27632. After 7 days of the cultivation, culture media in the culture inserts were discarded, and air-lifting cultivation was carried out in KCM without Y-27632. After 10 days of cultivation, the keratinocytes were harvested from the culture inserts as stratified epidermal equivalents by surgical forceps and fixed with 20% formalin solution.

2.7. Histological analysis for the epidermal equivalents

The fixed epidermal equivalents of cultured keratinocytes derived from hESCs and postnatal epidermis by air-lifting culture were routinely processed into 5 μm thick paraffin-wax-embedded sections and haematoxylin and eosin (HE) staining was performed using a standard protocol. Expressions of E-cadherin (CDH1), pan-keratins (KRTs), KRT14, keratin10 (KRT10), IVL, TP63, and vimentin (VIM) were analyzed by immunohistological methods. The sections were treated with mouse monoclonal anti-

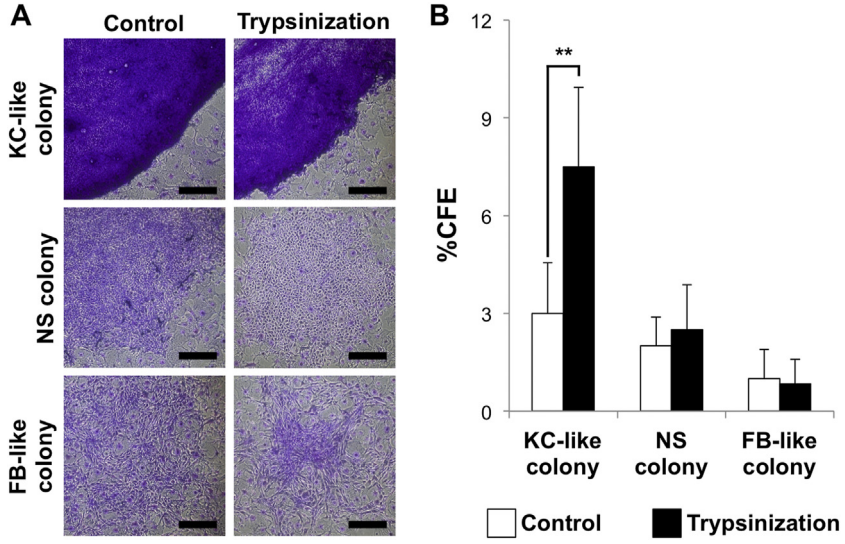

Fig. 2. Clonogenicity of human embryonic stem cell (hESC)-derived keratinocytes. (A) Morphologies of the colonies derived from hESC-derived keratinocytes in control (left column) and trypsinization group (right). Three types of colonies were observed with microscope and classified as keratinocyte (KC), non-stratified epithelial (NS), and fibroblast (FB)-like colonies. Scale bars indicate 500 μm. (B) Percentage of colony-forming efficiency (%CFE) of hESC-derived keratinocytes in the control and trypsinization groups. The percentages were calculated for each of the three types of colonies and compered between control and trypsinization groups. Open bars indicate %CFE of control group and closed bars indicate trypsinization group. **: $p < 0.01$.

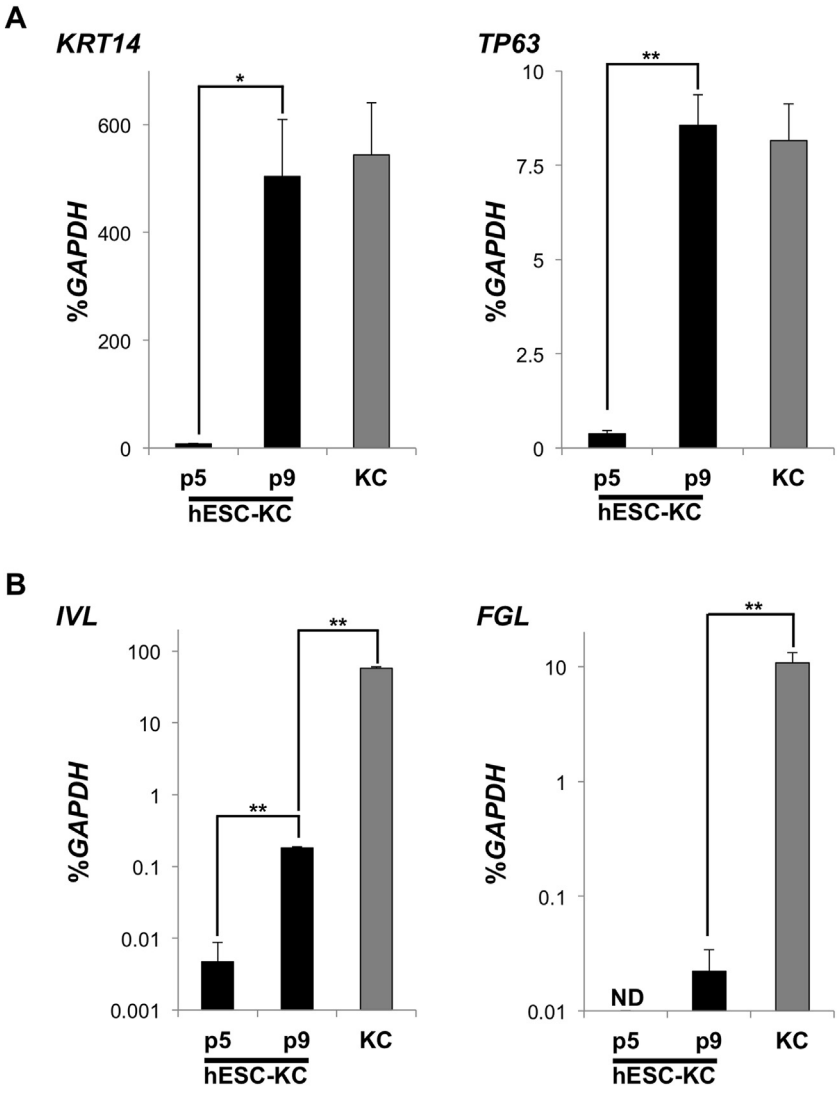

Fig. 3. Gene expression analysis of hESC-derived keratinocytes. Cultured human epidermal keratinocytes (KC) are used as positive controls for the qRT-PCR analysis. (A) Marker gene expressions of keratinocyte progenitor cells in hESC-derived keratinocytes (hESC-KC) at passage 5 and 9 (p5 and p9, respectively). The gene expressions of keratin 14 (*KRT14*) and tumor protein p63 (*TP63*) are presented as means of percent of glyceraldehyde-3-phosphate dehydrogenase expression (*%GAPDH*) with standard deviation. *: $p < 0.05$. **: $p < 0.01$. (B) Marker gene expressions of terminal differentiation of keratinocytes in hESC-KC at p5 and p9. The gene expressions of involucrin (*IVL*) and filaggrin (*FLG*) are presented as means of *%GAPDH* with standard deviation. Y-axis are shown as logarithm. ND: non-detection. **: $p < 0.01$.

CDH1 (1:100, 36/E-Cadherin, Becton Dickinson, NJ, USA), mouse monoclonal anti-KRTs (1:100 dilution, AE1/AE3, Thermo Fisher Scientific), mouse monoclonal anti-KRT14 (1:100, LL002, Abcam, Cambridge, UK), rabbit polyclonal anti-KRT10 (1:100, BioLegend, CA, USA), mouse monoclonal anti-IVL (1:1000, SY5, Sigma−Aldrich), mouse monoclonal anti-TP63 (1:100, 4A4, Abcam), and mouse monoclonal anti-VIM (1:100, Vim 3B4, Dako, Agilent Technologies, CA, USA) at room temperature for 90 min and were stained with peroxidase-conjugated secondary antibodies (Nichirei Bioscience, Tokyo, Japan) in accordance with the manufacture's suggested protocol. Human dermal and epidermal tissues were used for negative control of immnohistological analysis, and the results are shown in Supplemental Fig. 2.

2.8. Statistical analysis

Unpaired t-tests with Bonferroni correction were performed for clonogenicities of keratinocyte, non-stratified epithelial cells, and fibroblast-like cells ($n = 6$) in control and trypsinization groups. Gene expressions of hESC-derived keratinocyte at passage 5 and 9 and postnatal epidermal keratinocytes were analyzed by unpaired t-test with Bonferroni correction ($n = 3$). Cell densities of hESC-derived cells were measured for confirming effects of culture conditions and analyzed by unpaired t-tests with Bonferroni correction ($n = 3$). Doubling times of hESC-derived keratinocytes with or without Y-27632 were also evaluated by unpaired t-test ($n = 3$).

3. Results

3.1. Separation of hESC-derived keratinocytes

Keratinocytes were isolated from hESC-derived differentiated cells using sensitivity to trypsin before serial cultivation (Fig. 1A). Cellular morphologies before and during the trypsinization are shown in Supplemental Fig. 1. Upon trypsin treatment, the proportion of keratinocytes increased as serial culture progressed, compared to the control group (Fig. 1B). Cells collected in 1st trypsinization at passage 6 (PDL2.0) were cultured in the serial

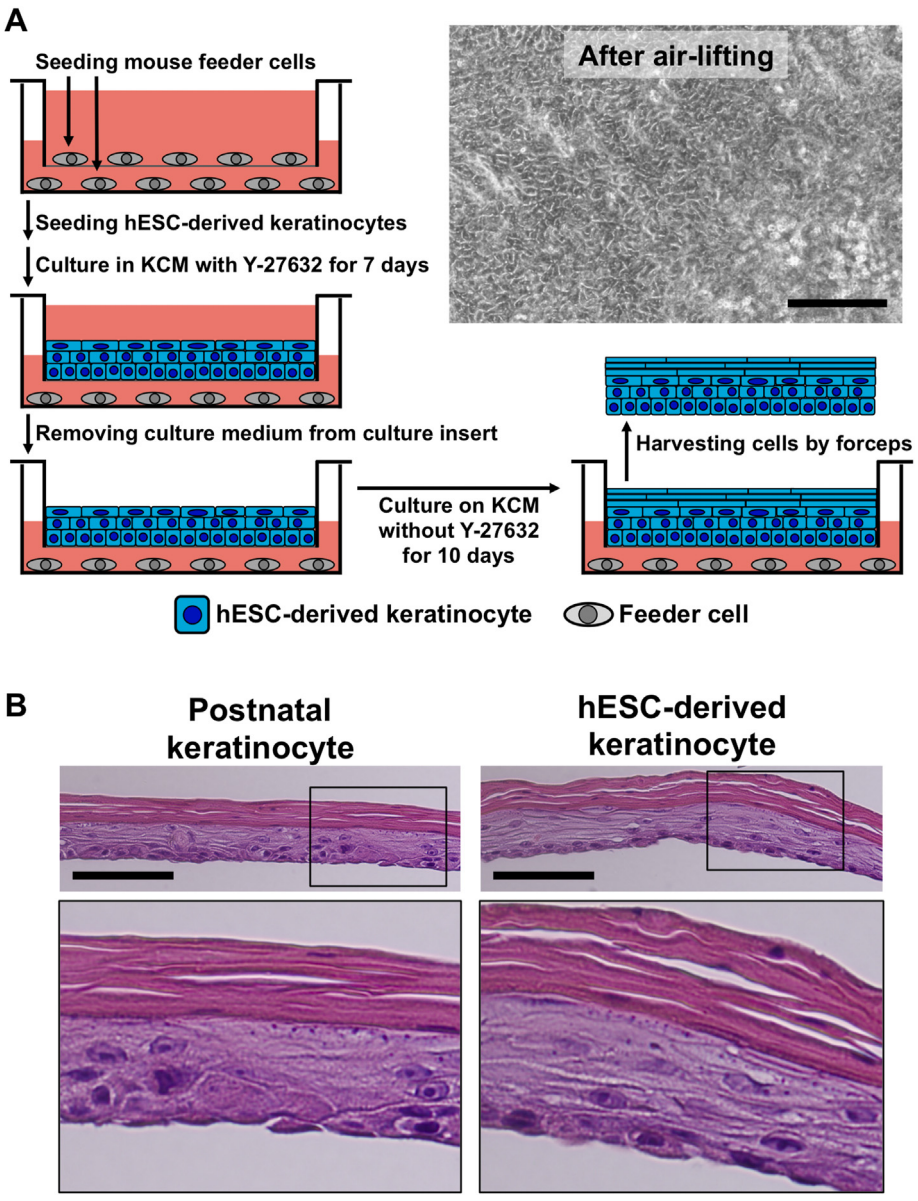

Fig. 4. Air-lifting culture of human embryonic stem cell (hESC)-derived keratinocytes. (A) Schematic illustration of air-lifting culture method for fabricating stratified epidermal equivalents. Molphology of hESC-derived keratinocytes after air-lifting culture is shown. (B) Paraffin sections of the equivalents were stained with haematoxylin and eosin. Scale bars indicate 200 μm.

culture condition and in conventional culture method for fibroblast by using α-MEM supplemented with FBS (Fig. 1C). Because keratinocytes do not proliferate in the conventional fibroblast culture, proliferative cells observed in the α-MEM supplemented with FBS are classified as proliferative non-keratinocyte cells. These results indicate that additional trypsinization is useful for separating keratinocytes from other differentiated hESCs. To confirm the effect of trypsinization, the clonogenicities of the control and trypsinization groups were analyzed at passage 7 (PDL3.7). During cultivation, there were three types of colonies, classified as keratinocyte, non-stratified epithelial, and fibroblast-like colonies, observed in both groups (Fig. 2A). The colony-forming efficiency of keratinocytes in the trypsinization group was significantly higher than in the control group (Fig. 2B). Although fibroblast-like cells were not observed during serial cultivation of the trypsinization group at passage 7 (PDL3.7) (Fig. 1B), such colonies were observed in both groups (Fig. 2A). To confirm the presence of proliferative non-keratinocyte cells after more serial cultivation, the hESC-derived keratinocytes at passage 13 (PDL23) were cultured on normal culture dishes without the mouse feeder layer in α-MEM supplemented with 10% FBS. Only hESC-derived keratinocytes were observed upon serial cultivation, and no proliferative non-keratinocyte cells were observed in the α-MEM (Fig. 1D). These results indicate that trypsinization is useful for isolation of keratinocytes from differentiated hESCs. The serial culture conditions did indeed decrease the proliferation of non-keratinocyte cells in the hESC-derived keratinocytes.

3.2. Gene expressions of hESC-derived keratinocytes

Because clonogenicity of hESC-derived keratinocytes was demonstrated, gene expression analysis was performed to confirm whether hESC-derived keratinocytes contained progenitor cells of keratinocyte by qRT-PCR. The gene expression analysis

demonstrated that expressions of *KRT14*, known as marker of basal cells of epidermal tissue, and *TP63*, marker of progenitor cell marker of keratinocyte, in hESC-derived keratinocyte at passage 9 were significantly higher than the cells at passage 5 (Fig. 3A). Interestingly, there were no significant difference between the gene expressions of *KRT14* and *TP63* in hESC-derived keratinocytes at passage 9 and cultured human epidermal keratinocytes at passage 3. Gene expressions of terminal differentiation marker of keratinocyte, *IVL* and *FGL*, in hESC-derived keratinocytes at passage 9 were significantly lower than in epidermal keratinocytes. The results of gene expression analysis indicate that progenitor cells of keratinocyte are enriched in hESC-derived keratinocytes by trypsinization and serial cultivation, and terminal differentiation of the cells is suppressed in the serial culture condition by using serum-free low [Ca^{2+}] medium and Y-27632.

3.3. Stratified epidermal equivalent with hESC-derived keratinocytes

hESC-derived keratinocytes were successfully separated from the differentiated hESCs by additional trypsinization and serial cultivation. To confirm the differentiation potency of the hESC-derived keratinocytes into stratified epidermal keratinocytes with terminal differentiation, the keratinocytes were seeded onto cell culture inserts for air-lifting culture (Fig. 4A). Histological analysis for the air-lifting cultures showed that hESC-derived keratinocytes formed stratified epidermal equivalents with cornified layers, similar to postnatal epidermal keratinocytes (Fig. 4B). The epithelial cell markers CDH1 and KRTs were expressed in the keratinocytes derived from hESCs and epidermal tissues, and a mesenchymal cell marker, VIM, was not expressed (Fig. 5). Additionally, hESC- and epidermis-derived keratinocytes expressed not only the progenitor cell markers KRT14 and TP63, but also the terminal differentiation markers, KRT10 and IVL (Fig. 5). The percentage of TP63-positive cells in the hESC-derived kerationcytes and postnatal epidermal keratinocytes was 36.7 ± 3.0 and 33.0 ± 5.4 (mean ± SD), respectively, implying that there is no significant difference between the two types of keratinocytes. There results indicate that the differentiation potency of hESC-derived keratinocytes remains unchanged during long-term cultivation under the experimental settings of this study.

3.4. Effect of Y-27632 and LN-511-E8 on hESC-derived keratinocytes

After isolation by trypsinization, hESC-derived keratinocytes were serially cultured with a mouse feeder layer on culture dishes coated with LN-511-E8 in DK-SFM supplemented with Y-27632. Morphologies of the hESC-derived keratinocyte at passage 5

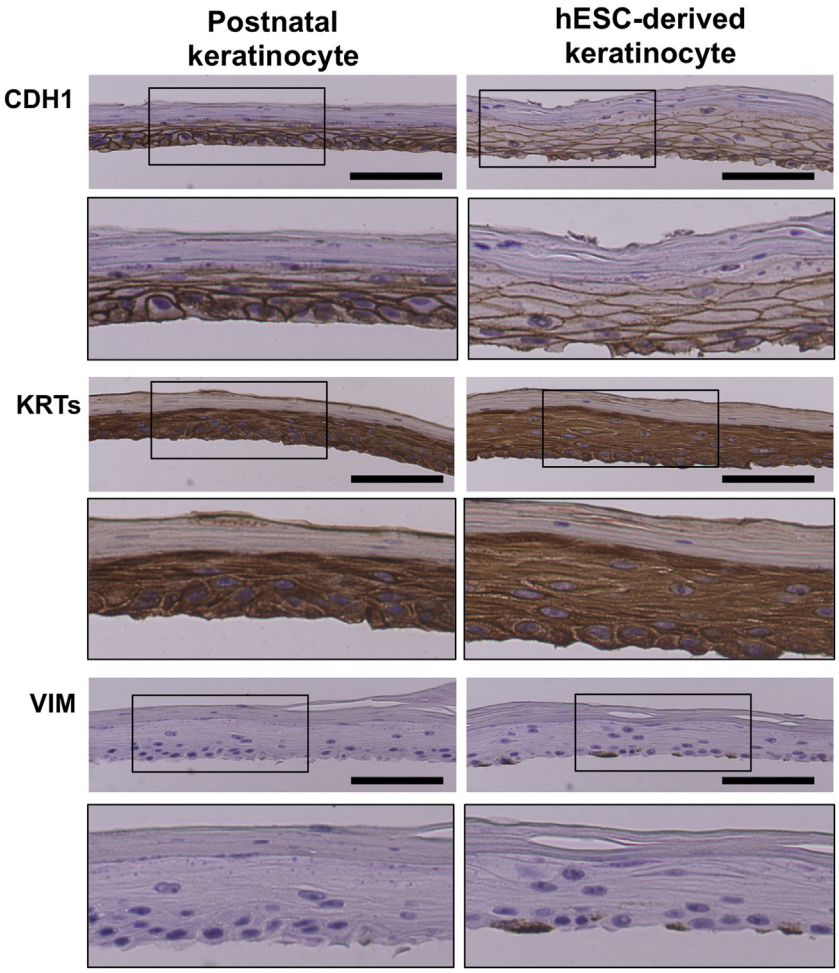

Fig. 5. Expression of markers in epidermal equivalents derived from hESC-derived keratinocytes. Fabrication of epidermal equivalents were carried out by the air-lifting culture method with postnatal human epidermal keratinocytes (left column) and hESC-derived keratinocytes (right column), and the equivalents were analyzed by immunohistochemical method to confirm expression of E-cadherin (CDH1), pan-keratins (KRTs), vimentin (VIM), keratin14 (KRT14), tumor protein p63 (TP63), keratin10 (KRT10), and involucrin (IVL). Scale bars indicate 200 μm.

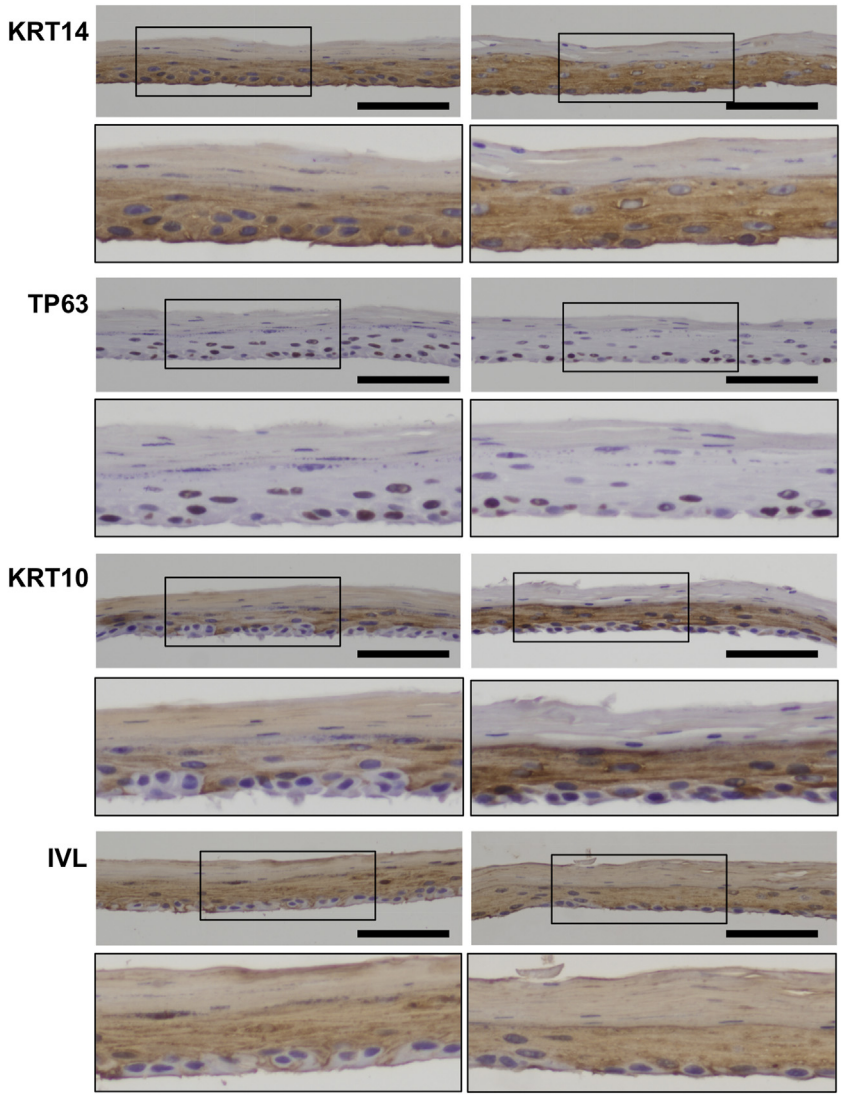

Fig. 5. (*continued*).

(PDL0), passage 11 (PDL16), passage 16 (PDL31), and passage 22 (PDF45) are shown in Fig. 6A. PDL from passage 5 (PDL0) to 22 (PDL45) and doubling times from passage 7 (PDL3.7) to 21 (PDL44) were calculated (Fig. 6B and C). Subcultured hESC-derived keratinocytes exhibited a logarithmic growth phase until passage 19 (PDL41), and doubling time during the growth phase was 2.3 ± 0.14 days (mean ± SD). The results indicate that LN-511-E8 and Y-27632 have possibilities as crucial supplements for facilitating long-term cultivation. Therefore, five culture conditions for hESC-derived keratinocytes were evaluated by numbers of cellular density after the cultivations and doubling times of the cells for confirming necessity of LN-511-E8, Y-27632, and feeder layer for maintaining the proliferation activity (Fig. 7). The five culture conditions and morphologies of hESC-derived keratinocytes under the conditions are shown in Fig. 7A and B. After these cultivations, cell densities of hESC-derived keratinocytes under culture condition 2, 3, 4 and 5 were significantly lower than density of hESC-derived keratinocytes under condition 1 (Fig. 7C). Doubling times of hESC-derive keratinocytes under culture conditions 1 and 2 were 1.8 ± 0.026 and 5.4 ± 0.53 days (mean ± SD), respectively, and the doubling time under condition 2 was significantly higher than that of condition 1 (Fig. 7D). These results suggest that LN-511-E8 and Y-27632 are essential for proliferation of hESC-derived keratinocytes that are co-cultured with feeder layer and subjected to subsequent long-term cultivation.

4. Discussion

Movahednia et al. defined two approaches for preparation of keratinocytes from hESCs: (i) spontaneous differentiation of the cell linage, (ii) direct differentiation by using growth factors, cytokines, and microenvironments [12]. Interestingly, we found that a portion of the hESCs spontaneously differentiated into keratinocytes under culture conditions for inducing differentiation into parenchymal hepatocytes from pluripotent stem cells. In the present study, although the mechanism for the spontaneous differentiation into keratinocyte is unknown, the keratinocytes were successfully isolated from the differentiated hESCs by serial cultivation with additional trypsinization. Indeed, because serial cultivation was implemented using serum-free low Ca^{2+} concentration medium in which cellular adhesion and proliferation of fibroblasts were suppressed, number of non-keratinocyte cells decreased during serial

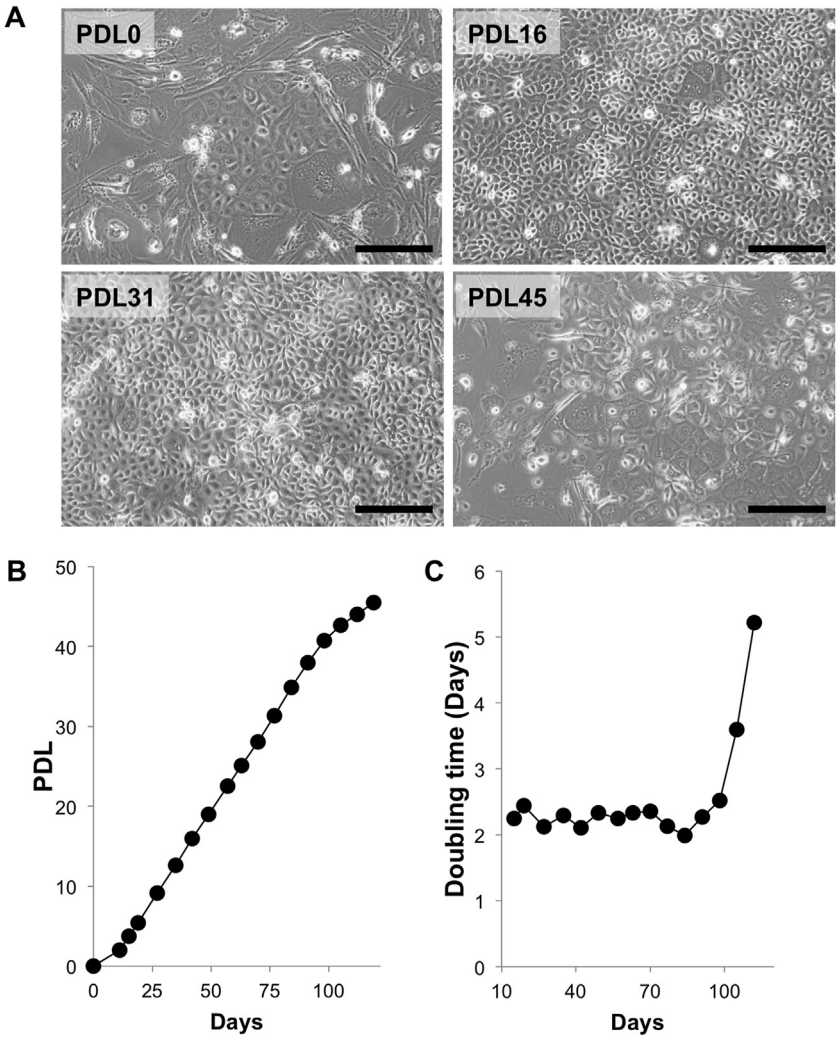

Fig. 6. Result of serial cultivation of hESC-derived keratinocytes. Population doubling level (PDL) of hESC-derived keratinocyte was calculated from passage 5 (PDL0) to 22 (PDF45). PDL of the keratinocytes at passage 5 before separation by trypsinization was defined as 0. (A) Morphologies of the keratinocyte during this serial cultivation are shown at passage 5 (PDL0), passage 11 (PDL16), passage 16 (PDL31), and passage 22 (PDF45). Scale bars indicate 200 μm. (B) Proliferation curve of hESC-derived keratinocyte during the serial cultivation is indicated by PDL from passage 5 (PDL0) to 22 (PDL45). (C) Doubling times of hESC-derived keratinocytes during the serial cultivation were calculated from passage 7 (PDL3.7) to 21 (PDL44). Mean of a doubling time was 2.3 days (SD 0.14) during the logarithmic growth phase from passage 7 (PDL3.7) to 19 (PDL41).

cultivation. These results indicate that isolation method of hESC-derived keratinocytes shown in the present study is simple and useful for enrichment of hESC-derived keratinocytes from other differentiated hESCs.

In previous study, frequency of IVL expression is reduced in immortalized keratinocyte differentiated from hESCs by transduction with the E6E7 gene compared with keratinocytes cultured from postnatal human epidermis [14]. Interestingly, although keratinocytes derived from hESCs are immortalized by transduction with human BMI1 gene for suppressing expression of cyclin dependent kinase inhibitor 2A (CDKN2A) by which senescence of postnatal keratinocytes are induced, morphology and expression of differentiation markers in organotypic culture of the immortalized keratinocytes differ from normal and immortalized postnatal keratinocytes [31–33]. In the present study, differentiation potency of the isolated hESC-derived keratinocytes was confirmed by air-lifting culture and immunohistological analysis. Moreover, the proliferative lifespan of the hESC-derived keratinocytes was maintained for approximately one hundred days, and the PDL of the keratinocytes was expanded to more than 40. These results indicate that the hESC-derived keratinocytes isolated in the present study have proliferative activity and potency of differentiation to form stratified epidermal equivalent similar to epidermal keratinocytes. In our previous study, cell lines of hESC with clinical grade were established for preparing cellular products to apply human patients [26], and in the present study, the hESC line was used for preparing keratinocytes with potencies of proliferation, clonogenicity, and differentiation into mature epidermal tissue *in vitro*. Therefore, although mouse feeder layer is required for maintaining serial culture of hESC-derived keratinocyte and fabrication of transplantable epidermal grafts of the keratinocytes, the hESC-derived keratinocytes have a potential as allogeneic cellular products for regeneration of stratified epithelial tissues, such as skin, oral mucosa, and esophagus, similar to previous keratinocyte grafts by using mouse 3T3 feeder layer in clinical situations [1,2].

Previous studies have demonstrated that hESC-derived keratinocytes show markedly lower proliferative activity than postnatal keratinocytes. On the other hands, inhibition of Rho kinase effectively expands the proliferative lifespan of postnatal keratinocytes without infection by HPV [17]. Moreover, proliferation and clonogenicity of keratinocytes indicate dose dependency of Y-27632, and

A

No. of culture conditions	1	2	3	4	5
LN511-E8 (2.5 μg/mL)	+	+	-	-	+
Y-27632 (10 μmol/L)	+	-	+	-	+
Feeder (2.5x10^4/cm^2)	+	+	+	+	-
Cultivation (Days)	7	12	12	12	12

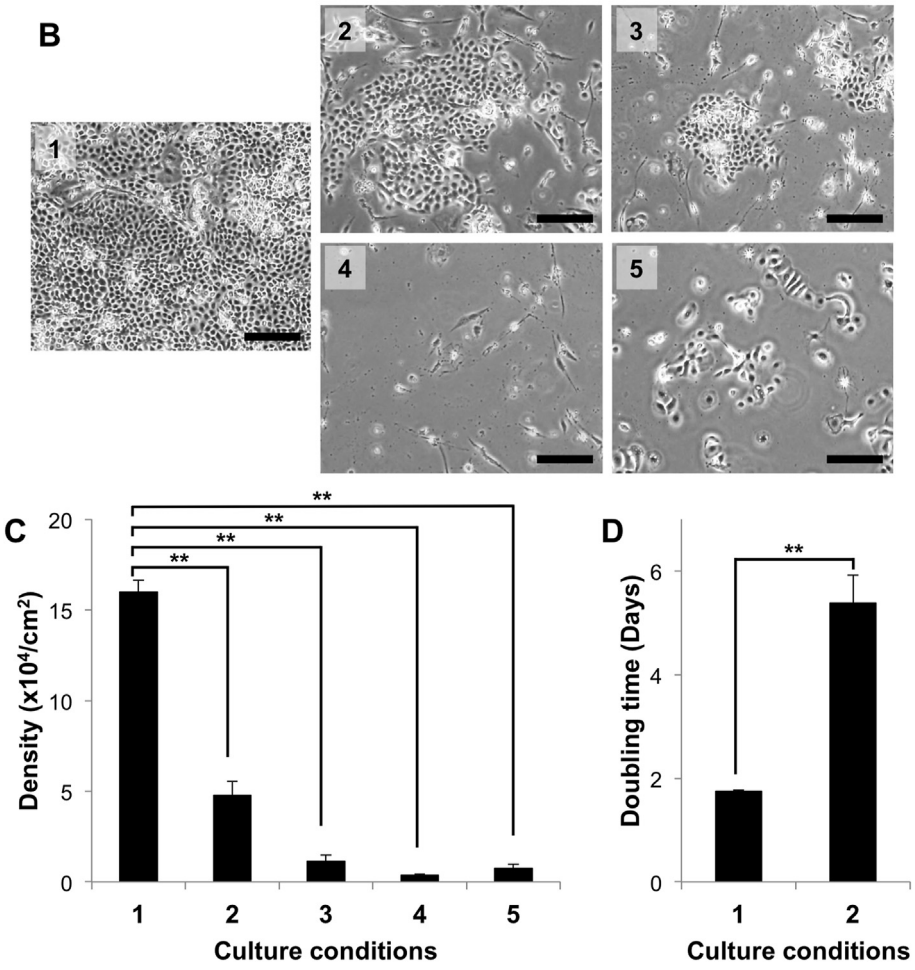

Fig. 7. Laminin-511 E8 fragment (LN-511-E8) and Y-27632 are essential for serial cultivation of hESC-derived keratinocytes. hESC-derived keratinocytes were cultured in five culture conditions for confirming efficacy of LN-511-E8, Y-27632, and the feeder layer for proliferation of keratinocytes. (A) The five culture conditions are shown in this table. (1) Culture condition 1 was similar to serial cultivation with Y-27632, LN-511-E8, and the mouse feeder layer for hESC-derived keratinocytes. (2) Culture condition 2 was identical to condition 1 except that Y-27632 was removed. (3) In culture condition 3, LN-511-E8 was removed from condition 1. (4) In culture condition 4, the keratinocytes were co-cultured with a mouse feeder layer without Y-27632 and LN-511-E8. (5) In culture condition 5, the keratinocytes were cultured with LN-511-E8 and Y-27632 without the feeder layer. (B) Cellular morphologies of hESC-derived keratinocytes under the five culture conditions. Scale bars indicate 200 μm. (C) Cellular densities of hESC-derived keratinocytes after cultivations under the five conditions. **: $p < 0.01$. (D) Doubling times of hESC-derived keratinocytes cultured in condition 1 and 2. **$p < 0.01$.

the proliferation is also expanded by treatment with reagents with inhibition activity to Rho-kinase, such as fasudil hydrochloride, HA1000 hydrochloride, and GSK 429286 [16–18,34]. Transcriptome analysis of epidermal keratinocytes reveals that Y-27632 up-regulates gene expression associated with cell cycle and division and down-regulates expression of genes promoting epithelial cell differentiation and keratinization [34]. The suppression of terminal differentiation is thought to be one of the mechanisms underlying extension of the proliferative lifespan of postnatal keratinocytes by Rho kinase inhibition. On the other hand, although Y-27632 and a mouse feeder layer were used for serial cultivation of hESC-derived keratinocytes, culture surfaces coated with LN-511-E8 were required for maintaining the proliferative ability of hESC-derived keratinocytes. Due to requirement of LN-511-E8 for expansion of replicative lifespan of hESC-derived keratinocytes, LN-511-E8 may be important to elucidate mechanism for expanding the proliferative lifespans of hESC-derived keratinocytes by inhibition of Rho kinase and for difference of proliferative lifespan between hESC-derived keratinocytes and postnatal keratinocytes.

In conclusion, hESC-derived keratinocytes were successfully isolated and expanded without transduction of any exogenous genes by serial cultivation with a feeder layer in serum-free low Ca^{2+} medium shown in the present study. Under these conditions, LN-511-E8 and Y-27632 were required for maintaining the proliferative activity of hESC-derived keratinocytes. These results suggest that a combination of LN-511-E8 and Y-27632 will be essential

supplements for the fabrication of epidermal equivalents from pluripotent stem cells for clinical applications.

Declaration of competing interest

AU is a co-researcher with MTI Ltd., Terumo Corp., BONAC Corp., Kaneka Corp., CellSeed Inc., ROHTO Pharmaceutical Co., Ltd., SEKISUI MEDICAL Co., Ltd., Metcela Inc., PhoenixBio Co., Ltd., Dai Nippon Printing Co., Ltd., Twocells Co., Ltd. AU is a stockholder of TMU Science Ltd., Morikuni Ltd., tbt Co., Ltd., and Japan Tissue Engineering Co., Ltd. The other authors declare that there is no conflict of interest regarding the work described herein.

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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.2021.07.004.

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