



# Establishment of Immortalized Primary Human Foreskin Keratinocytes and Their Application to Toxicity Assessment and Three Dimensional Skin Culture Construction

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## Abstract

In spite of frequent usage of primary human foreskin keratinocytes (HFKs) in the study of skin biology, senescence-induced blockage of *in vitro* proliferation has been a big hurdle for their effective utilization. In order to overcome this passage limitation, we first isolated ten HFK lines from circumcision patients and successfully immortalized four of them via a retroviral transduction of high-risk human papillomavirus (HPV) E6 and E7 oncogenes. We confirmed expression of a keratinocyte marker protein, keratin 14 and two viral oncoproteins in these immortalized HFKs. We also observed their robust responsiveness to various exogenous stimuli, which was evidenced by increased mRNA expression of epithelial differentiation markers and pro-inflammatory genes in response to three reactive chemicals. In addition, their applicability to cytotoxicity assessment turned out to be comparable to that of HaCaT cells. Finally, we confirmed their differentiation capacity by construction of well-stratified three dimensional skin cultures. These newly established immortalized HFKs will be valuable tools not only for generation of *in vitro* skin disease models but also for prediction of potential toxicities of various cosmetic chemicals.

**Key Words:** Human foreskin keratinocyte, Immortalization, Toxicity assessment, Three-dimensional skin culture

## INTRODUCTION

Skin serves as a first-line of defense against various exogenous chemicals as well as infectious pathogens. Dermis and epidermis, two major components of skin, exerts most of its protective effects against outside assaults. More than 95% of the epidermal tissue is composed of one type of cells, which are keratinocytes. While keratinocytes at basal layer of the epidermis are able to support continuous cell division as stem cell populations, those at supra-basal layer of the epidermis exits from cell cycle and are committed to sequential differentiation. Due to their essential roles in skin biology, primary human foreskin keratinocytes (HFKs) have been used not only for pathogenesis study of skin-related diseases but also for toxicity assessment of various cosmetics agents.

In general, primary HFKs are able to support 15 to 20 population doublings in typical serum-free culture media *in vitro* (Stoppler *et al.*, 1997; Kiyono *et al.*, 1998). Once they reach cell proliferation limit, they enter a unique cellular state called "senescence" (Hayflick and Moorhead, 1961). During this senescence period, primary HFKs first stop responding to exogenous mitogenic stimuli. They also increase cellular adhesion to extracellular matrix. Other senescence-induced changes include bigger and flattened cell morphology, enhanced lysosomal biogenesis (Shelton *et al.*, 1999; Serrano and Blasco, 2001; Narita *et al.*, 2003; Ben-Porath and Weinberg, 2004, 2005), formation of multiple nuclei (Stewart and Weinberg, 2002), and generation of heterochromatic foci (Fridman and Tainsky, 2008). Short lifespan of primary HFKs has hindered their utilization as a model system to study skin biology *in vitro*.

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In order to avoid this cell passage problems, several spontaneously immortalized keratinocyte cell lines such as NM1 (Baden *et al.*, 1987), HaCaT (Boukamp *et al.*, 1988), and NIKS (Allen-Hoffmann *et al.*, 2000) have been established and extensively used. However, these immortalized keratinocyte cell lines have been shown to possess several undesirable genetic defects including p53 mutations (Lehman *et al.*, 1993) and incorrect chromosomes numbers (Allen-Hoffmann *et al.*, 2000). Therefore, there has been a consistent need to establish immortalized keratinocytes with characteristics of normal human keratinocytes.

Artificial immortalization of primary HFKs can be achieved by four different methods (Choi and Lee, 2015). They include overexpression of telomerase, inactivation of cell cycle regulatory genes, inhibition of a specific host kinase, and overexpression of viral oncogenes. Cell cycle regulatory genes such as p16<sup>INK4A</sup>, pRb, p14<sup>ARF</sup>, p53, and p21<sup>CIP1</sup> are representative inactivated genes for immortalization. Cellular oncogenes such as c-MYC and Bmi-1 were also frequently inactivated for cellular immortalization. In case of viral oncogenes, T antigen from simian virus 40 (SV40), E6 and E7 from human papillomavirus (HPV), and E1A and E1B from adenovirus are the best-characterized ones with potential to immortalize host cells. In particular, high-risk type human papillomavirus (HPV) E6 and E7 are the most frequently employed viral oncogenes for immortalization purpose due to their specific activities to target two key tumor suppressor genes, p53 and pRb for degradation, respectively (Choi and Lee, 2015). In addition, endogenous features of tissues have been shown to be better preserved in E6 and E7-immortalized cell lines than those immortalized by other methods (Durst *et al.*, 1987; Hawley-Nelson *et al.*, 1989; Flores *et al.*, 1999).

In order to study differentiating functions of epithelial tissues of a normal skin *in vitro*, a specially designed differentiation technique needs to be applied to primary HFKs (Choi and Lee, 2015). In this differentiation technique, primary HFKs are designed to grow on top of a dermal substitute while maintaining the air-liquid interface, which was shown to be a critical driving factor for efficient epithelial differentiation and stratification. Several companies have developed different types of three-dimensional (3D) skin culture systems and applied them to irritancy and corrosiveness test of cosmetic materials in the animal-free setting. However, due to the limited supply of primary HFKs, prices of these commercialized 3D skin culture products are relatively high. Therefore, cost-effective and stable supply of human keratinocytes will be very helpful to provide 3D skin culture products with more affordable prices.

In order to overcome this endogenous limitation of primary HFKs, we immortalized primary HFKs by retroviral expression of papillomavirus (HPV) E6 and E7 oncogenes. We were able to confirm their robust responsiveness to various exogenous stimuli, comparable applicability to cytotoxicity assessment, and superior differentiation capacity. We strongly believe that these newly-generated immortalized HFKs will be a valuable tool to study many aspects of skin biology in the *in vitro* setting.

## MATERIALS AND METHODS

### Isolation of primary HFKs

Eight primary HFK lines were isolated from human foreskin

biopsies samples provided by Dongguk University Hospital (Goyang, Korea) after IRB approval by Dongguk University IRB committee on Nov 27 2014 with approval number of 2014-87. Three additional primary HFK lines were purchased from two commercial vendors in Korea (Biosolution, Seoul, Korea and Tego Science, Seoul, Korea). Primary HFKs was isolated as previously described (Lee and Laimins, 2004; Lee *et al.*, 2007). Briefly, the skin specimen was washed several times with PBS buffer until complete clearance of contaminated bloods on samples. After adipose tissues were trimmed by using scissors and forceps, remaining samples were cut into small pieces. These small pieces were plated onto a 10 cc culture dish containing dispase II (2.4 U/ml, Roche, Basel, Switzerland) and incubated for overnight at 4°C. Next day, epidermal tissues were separated from dermal tissues by using forceps. The separated epidermal tissues were placed on 0.25% trypsin (Hyclone, Little Chalfont, UK)-containing 6 mm dish for 10 min. Then, the epidermal tissues were dissected as fine as possible by using forceps. After a brief spin down, isolated HFKs were resuspended and incubated with appropriate volume of KGM-Gold media (Lonza, Basel, Switzerland).

### Cell culture

Primary and immortalized HFKs were cultured in KGM-Gold media (Lonza). NIH3T3 and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone) supplemented with 1% L-glutamine (Hyclone), 1% penicillin/streptomycin (Hyclone), and 10% fetal bovine serum (JRSscientific Inc., CA, USA) at 37°C with 5% CO<sub>2</sub>. Typically, when cells reach around 90% confluency, they were split to 1:5 dilution ratio and their cell passage goes up by one.

### Plasmids and chemical reagents

pLXSN-16E6E7 and retroviral packaging vectors were gifts from Dr. Denise Galloway from Fred Hutchinson Cancer Research Center, Seattle, WA, USA (Halbert *et al.*, 1991). All of the chemical reagents used for toxicity assessment were purchased from Sigma (St. Louis, MO, USA).

### Immortalization of primary HFKs

In order to establish immortalized HFKs, retroviral particles encoding HPV16E6 and E7 genes were produced by using a retroviral system as previously described (Choi *et al.*, 2014). Briefly, pLXSN-HPV16E6E7 plasmid and two packaging vectors (Gag-Pol and VSV-G) were transfected into 293T cells. Next day, cell culture media was changed with fresh DMEM containing 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. After 24 hr, the media containing retroviral particles was harvested. After a brief spin down of debris, supernatant was filtered with 0.45 µm filter (Sartorius, Gottingen, German). Before transduction, HFKs were seeded at a cell density of 2×10<sup>5</sup> cells/cm<sup>2</sup> in 6 cm dishes. Retroviral supernatants and fresh KGM media were mixed by 1:1 ratio in the presence of 10 µg/ml of hexadimethrine bromide (Polybrene; Sigma) and then HFKs media was changed with retroviral media. At following day, retroviral media was removed and then fresh KGM media was added into infected HFKs. After 3 days, selection of immortalized cells was started with G418 200 µg/ml (Sigma) for more than 7 days, after which only infected cells survived.

### PCR analysis

In order to extract genomic DNAs from HFKs and immortal-

ized HFKs, purelink® genomic DNA kits (Invitrogen, Carlsbad, CA, USA) was used by following the manufacturer's protocol. PCR was performed with HPVE6E7 region primer: forward 5'-CTA GCT AGC ATG CAC CAA AAG AGA ACT GC-3' and reverse 5'-CCG GAA TTC TTA TGA TGG TTT CTG AGA ACA GAT GGG-3'. PCR was carried out for 28 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min. Generated PCR products were analyzed by an agarose gel.

### Western blot analysis

Cells were lysed in RIPA buffer (150 mM NaCl, 1% SDS, 1% deoxycholic acid sodium salt, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA; Thermo, Waltham, MA, USA) containing a cocktail of protease and phosphatase inhibitors (Thermo). Protein concentration was determined by Bradford assay (Bio-rad, Hercules, CA, USA). Total cell protein (30 µg) was electrophoresed on an SDS-polyacrylamide gel. Then, samples were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, UK). Proteins of interest were detected by using following antibodies: anti-phospho Rb and keratin 14 (1:1,000 for phosphor Rb and 1:10,000 for keratin 14, NovusBio, CO, USA), anti-Rb (1:1,000 for Rb, Thermo), anti-p53 (1:1,000, Santa Cruz, CA, USA), anti-β-actin (1:10,000, Santa Cruz), horseradish peroxidase-conjugated anti-mouse, horseradish peroxidase-conjugated anti-Rabbit (1:10,000 for mouse and 1:20,000 for Rabbit; Thermo).

### RNA extraction and quantitative real time RT-PCR

Experiments to test responsiveness of immortalized HFKs to retinoic acid, hydroquinone, and formaldehyde were conducted as previously described (Cheong *et al.*, 2014; Lee *et al.*, 2016). Briefly, immortalized HFKs were incubated with designated compounds for 24 hr. Then, total RNA was isolated from immortalized HFKs using TRIzol™ (Invitrogen), according to the manufacturer's instructions. The concentration of RNA was determined spectrophotometrically, and the integrity of the RNA was assessed using a BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Two micrograms of RNA were reverse-transcribed into cDNA using SuperScript®III reverse transcriptase (Invitrogen) and aliquots were stored at -20°C. Quantitative real-time TaqMan RT-PCR technology (Q-RT-PCR) (Applied Biosystems, Foster City, CA, USA) was used to determine the expression level of selected target genes. The cycling conditions included a denaturing step at 95°C for 10 min and 50 cycles of 95°C for 15 sec and 60°C for 1 min. The TaqMan probes (Applied Biosystems) used in the Q-RT-PCR analysis were: filaggrin, Hs00856927\_g1; IL-8, Hs00174103\_m1; KRT10, Hs01043110\_g1; MMP-1, Hs00899658\_m1; and S100A7, Hs01923188\_u1. Human GAPDH (4333764F, Applied Biosystems) was also amplified to normalize variations in cDNA levels across different samples.

### Cytotoxicity assay (MTT)

Cells were seeded at 30,000 per well in 96-well plate (SPL) in triplicate. The cells were then treated with designated compounds. After 3 days incubation, cell viability assay was performed with EZ-CYTOX (10% tetrazolium salt; Dogen, Seoul, Korea) by following the manufacturer's instructions.

### Differentiation of immortalized HFKs

Primary and immortalized HFKs were cultured onto 100 mm dish to full confluency in KGM media (Lonza). After confirmation of cell density, cells were switched to differentiation induction media (1.8 mM CaCl<sub>2</sub> and 10% FBS). After 4 days and 7 days, pictures of differentiated cells were taken and their lysates were prepared further analysis.

### 3D skin culture without a dermal equivalent

3D epidermal skin equivalent composed of multilayered keratinocyte were reconstructed as previously described. Briefly, immortalized HFKs and HaCaT were seeded on a 12 mm Millicell™ (MerchMillipore, Darmstadt, Germany) and incubated 7 days to confluence. Next, epithelial differentiation was induced by air-liquid interface culture for 14 days with NIH3T3 feeder layers.

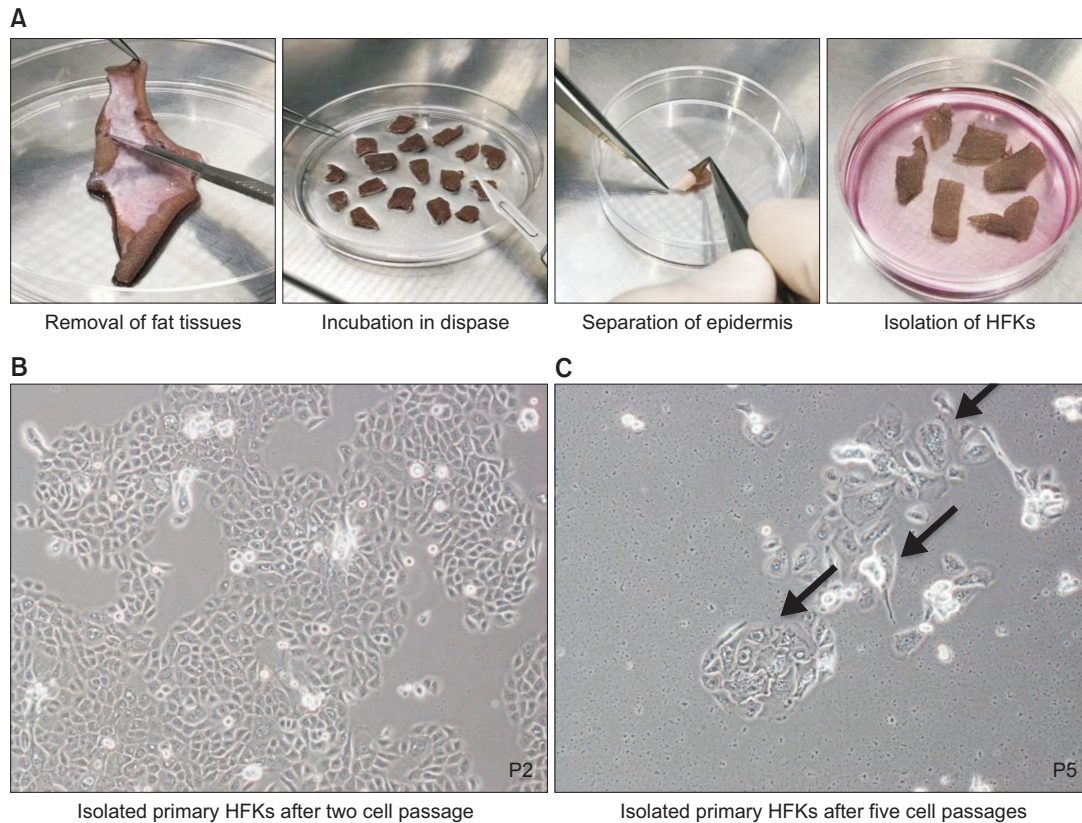
### Immunohistochemistry

The 3D epidermal skin equivalent were fixed with 10% formaldehyde, embedded with paraffin, and prepared as 0.4 µm sections using a RM2255 Microtome (Leica, Wetzlar, Germany). Immunohistochemistry was performed, using the avidin-biotin complex technique with Universal VECTASTAIN ABC Kit (Vector Laboratories, Burlingame, CA, USA). Paraffin sections were de-paraffinized in xylene, hydrated through a decreasing ethanol concentration grades, and endogenous peroxidase activity was quenched using 0.3% hydrogen peroxide in PBS. Non-specific binding was eliminated by incubating with diluted normal blocking serum. Sections were then serially incubated with primary antiserum diluted in buffer for 1 h, diluted biotinylated secondary antibody solution for 40 min, ABC reagent for 30 min, and peroxidase substrate solution until the desired stain intensity developed. All steps were performed at room temperature. For immunohistochemistry, we used monoclonal antibodies for cytokeratin 14 from Abcam (Cambridge, UK) and P63 from CHEMICON International Inc. (Temecula, CA, USA), Filaggrin from Novocastra Laboratories Ltd. (Newcastle, UK) and E-Cadherin from Santa Cruz Biotechnology Inc. Each section was counterstained with hematoxylin, mounted and entire tissue area was examined under on Olympus DX41 microscope (CenterValley, PA, USA).

### 3D skin cultures with a dermal equivalent

The method used to develop 3D skin cultures with a dermal equivalent was according to previously reported one (Regan and Laimins, 2013). Briefly, the dermal layer was made by mixing 10X DMEM, 10X reconstitution buffer, rat tail collagen I, and NIH3T3 cell. Its pH was adjusted by dropping 1 M NaOH until the color of mixture turned pink or red. The collagen mixture was poured onto 6 well plate, and then incubated at 37°C for 30 min. After formation of gel of collagen mixture, E-media (DMEM (4.5 mg/ml glucose) and Ham's F-12 mixed at ratio of 3:1 with 5 µg/ml insulin, 10 ng/ml EGF, 0.4 µg/ml hydrocortisone, 2×10<sup>-11</sup> M, 3,3',5-triiodo-L-thyronine, 5 µg/ml transferin, 10<sup>-10</sup> M cholera toxin, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS) was added into 6 well plate. At 2 or 3 days later, immortalized HFKs (1×10<sup>6</sup> cells) were plated gently onto the dermal layer. Media was changed at every day. After 2 or 3 days, the collagen gel was moved to a metal grid very carefully by using a spatula. Then, air-lift phase was subjected for 14 day with E-media containing 1.8×10<sup>-4</sup> M adenine and without EGF. After 14 days, the





**Fig. 1.** (A) How to isolate primary HFKs from patient foreskins. Four key steps were shown in pictures. Fatty tissues were removed from foreskin sample. Foreskin sample was cut into small pieces and incubated with dispase II. Epidermal part was separated from dermal part. Separated epidermal part was incubated with trypsin for isolation of HFKs. (B) Representative pictures of isolated primary HFKs after two cell passages. (C) Representative pictures of isolated primary HFKs after five cell passages. Arrows point to typical senescent cells.

sample was fixed by using 4% formaldehyde. Subsequently, dehydration, paraffin embedding and section, and hematoxylin and eosin stain was performed accordingly.

#### Histological analysis (H&E staining)

After sample was fixed with 4% formaldehyde, it went through serial dehydration, with 70%, 80%, 95%, 100% ethanol. Dehydrated samples were incubated to xylene, and then passed through paraffin penetration. After making paraffin blocks, paraffin block was sectioned into 10  $\mu\text{m}$  thickness samples. Next, this sample underwent rehydration, hematoxylin staining (Mayer's hematoxylin, Dako, CA, USA), and then eosin staining (Millipore).

#### Statistical analysis

All statistical analyses were performed with MINITAB1 software (Minitab Inc., State College, PA, USA). The statistical significance of the experimental data was analyzed using Student's *t* test. The results were expressed as the mean value with standard deviation. Data were regarded as significant if *p*-values were less than 0.05.

## RESULTS

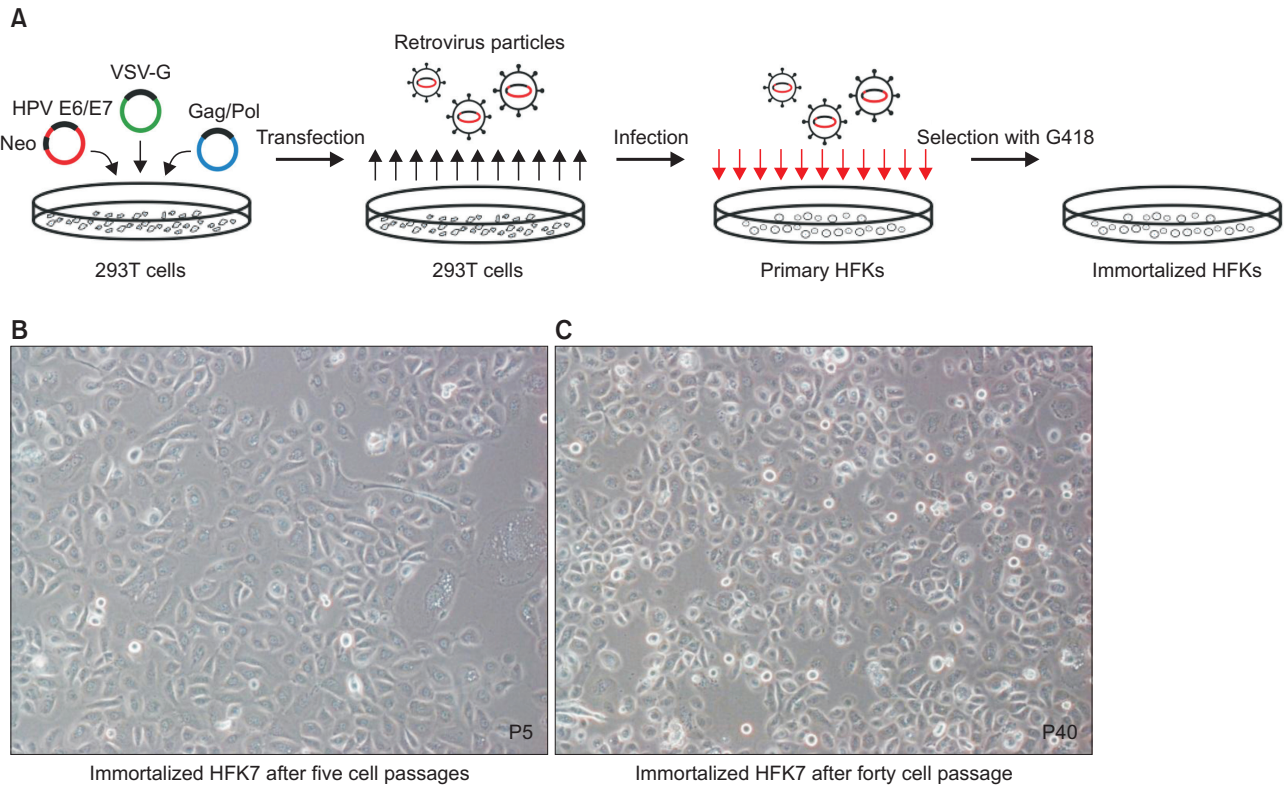
#### Isolation of primary HFKs from patients' foreskins

In order to establish primary HFKs cell lines, we first ob-

**Table 1.** Ten lines of primary HFKs used in this study, providers, and maximum passage number were listed

HFK number	HFK provider	Maximum passage number
HFK1	Dongguk Univ Hospital	8
HFK2	Dongguk Univ Hospital	7
HFK3	Dongguk Univ Hospital	8
HFK4	Dongguk Univ Hospital	6
HFK5	Dongguk Univ Hospital	8
HFK6	Dongguk Univ Hospital	7
HFK7	Dongguk Univ Hospital	8
HFK8	Commercial vendor A	1
HFK9	Commercial vendor A	2
HFK10	Commercial vendor B	4

tained circumcised foreskins from ten male patients at Dongguk University Hospital (Goyang, Korea) after IRB approval. By following a four step procedure shown in Fig. 1A, we were able to successfully isolate seven primary HFK lines from ten foreskin samples. They were labeled as HFK1 through HFK7 according to the order of isolation. We purchased three additional primary HFK lines from two commercial vendors (Table 1). They were also labeled as HFK8 through HFK10. A representative picture of isolated HFKs after two cell passages



**Fig. 2.** (A) A schematic diagram of how to establish immortalized HFKs by using a retroviral system. (B) Representative pictures of immortalized HFK7-E6E7 cells after five cell passages. (C) Representative pictures of immortalized HFK7-E6E7 cells after forty cell passages.

was shown in Fig. 1B. Both patients-derived and commercially-available primary HFKs were able to support around six to seven passages depending on cell lines. After seven cell passages, most of isolated HFKs started to slow down their cell proliferation and exhibited senescence-specific phenotypes, such as bigger cell size, enlarged nucleus, and elongated cell shape (Fig. 1C). All these late passage-associated changes led to a complete block in cell proliferation. Patients-derived HFKs was able to support four-to-five longer cell passages than commercially-available ones (Table 1).

**Immortalization of primary HFKs by using a HPV16-E6E7-encoding retrovirus**

In order to overcome this growth arrest imposed by senescence, we decided to immortalize primary HFKs by using a retroviral expression of HPV type 16 E6 and E7 oncogenes. As shown Fig. 2A, three retroviral packaging vectors encoding HPV16-E6E7 (pLXSN-HPV16-E6/E7), vesicular stomatitis virus glycoprotein (VSV-G), and retrovirus Gag/Pol were transfected into 293T packaging cells to generate HPV 16-E6E7-expressing retroviruses. Then, they were used to infect eight primary HFK lines, which were isolated previously. We did not include HFK8 and HFK9 cell lines for immortalization due to their severely limited cell proliferation in time of retroviral infection. Infected HFKs underwent neomycin selection for enrichment of HPV E6E7-expressing HFKs (Fig. 2B). Among eight HFK lines which were attempted for immortalization, four infected HFKs (HFK4, HFK6, HFK7, and HFK10) were able to support more than forty consecutive cell passages, which

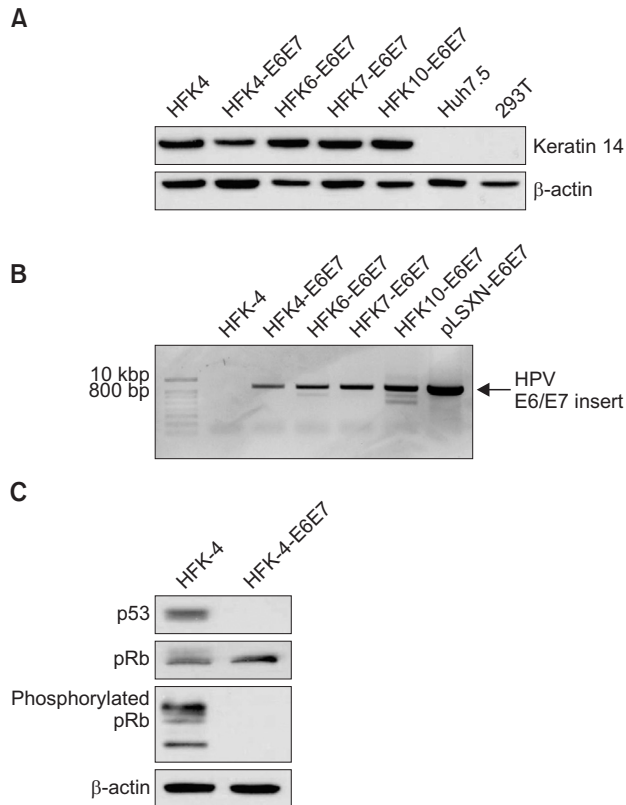
**Table 2.** Ten lines of primary HFKs used for retroviral infection, maximum passage number after infection, and immortalization status were listed. Successfully immortalized four cell lines were denoted with bold

HFK number	Retrovirus infection	Maximum passage number after infection	Immortalization
HFK1	Yes	<5	No
HFK2	Yes	<5	No
HFK3	Yes	<5	No
<b>HFK4</b>	<b>Yes</b>	<b>&gt;40</b>	<b>Yes</b>
HFK5	Yes	<5	No
<b>HFK6</b>	<b>Yes</b>	<b>&gt;40</b>	<b>Yes</b>
<b>HFK7</b>	<b>Yes</b>	<b>&gt;40</b>	<b>Yes</b>
HFK8	No	N/D	No
HFK9	No	N/D	No
<b>HFK10</b>	<b>Yes</b>	<b>&gt;40</b>	<b>Yes</b>

were generally accepted as a full immortalization without any sign of senescence or cellular crisis (Fig. 2C, Table 2).

**Confirmation of keratinocyte marker and HPV16 E6E7 proteins expression in immortalized HFKs**

Keratin 14 is one of the representative undifferentiated keratinocyte markers. Therefore, we wished to confirm continuous expression of keratin 14 in the immortalized HFKs. As shown in Fig. 3A, all immortalized HFKs (HFK4-E6E7, HFK6-E6E7, HFK7-E6E7, and HFK10-E6E7) were able to maintain



**Fig. 3.** Detection of keratinocyte marker and viral oncoproteins' expression (A) Confirmation of expression of a keratinocyte marker protein, keratin 14 in primary and immortalized HFKs by western blot analysis. (B) Confirmation of HPV-E6E7 DNAs in primary and immortalized HFKs. PCR was performed with primary and immortalized HFKs' genomic DNAs for detection of inserted HPV16-E6E7. pLXSN-E6E7 was used as a positive control. (C) Confirmation of functional expression of E6 and E7 proteins by western blot analysis for p53, pRb, and phosphorylated pRb.

expression of the keratin 14 like normal HFK4s. However, non-keratinocyte cell lines such as Huh7.5 (hepatocyte) and 293T (kidney cell) failed to express keratin 14 protein (Fig. 3A). In order to confirm insertion of HPV16 E6E7 DNAs into chromosomes of immortalized HFKs, we performed PCR analysis by using genomic DNAs isolated from normal and immortalized HFKs. A retroviral vector (pLXSN-E6E7) was used as a positive control for detection of HPV16 E6E7 gene. As shown in Fig. 3B, we were able to detect the inserted HPV16-E6E7 genes only in the immortalized HFKs, not in the normal HFKs. Western blot analysis further confirmed the functional expression of HPV E6 and E7 proteins in the immortalized HFKs (HFK4-E6E7), which was demonstrated by reduced expression of their respective target host proteins, p53 and phosphorylated pRb, respectively (Fig. 3C). Based on these results, we concluded that retroviral infection induced primary HFKs immortalization through HPV E6 and E7 expression.

### Characterization of responsiveness of immortalized HFKs to exogenous stimuli

After a successful establishment of immortalized HFKs by a retroviral transduction, we wished to test their behavioral similarity to that of normal HFKs. In general, normal HFKs in-

crease secretion of epithelial differentiation markers including A100A7, filaggrin, and keratin 10 upon retinoic acid treatment in order to strengthen skin barrier function, whereas this differentiation-induced protection is impaired by highly reactive chemicals such as hydroquinone and formaldehyde (Cheong *et al.*, 2014; Lee *et al.*, 2016). Especially, enhanced production of inflammatory cytokines in normal HFKs by formaldehyde was well characterized. As expected, retinoic acid treatment gave rise to increased secretion of S100A7 in immortalized HFK7-E6E7 cells. However, in contrast to our expectation, expression of both filaggrin and keratin 10 was reduced upon retinoic acid treatment (Fig. 4A). As expected, immortalized HFK7-E6E7 cells produced much higher levels of mRNAs for pro-inflammatory cytokine genes, such as TNF- $\alpha$ , IL-8, and MMP-1 in the presence of formaldehyde (Fig. 4B). These data indicates that immortalized HFKs maintain responsiveness to exogenous stimuli like normal HFKs.

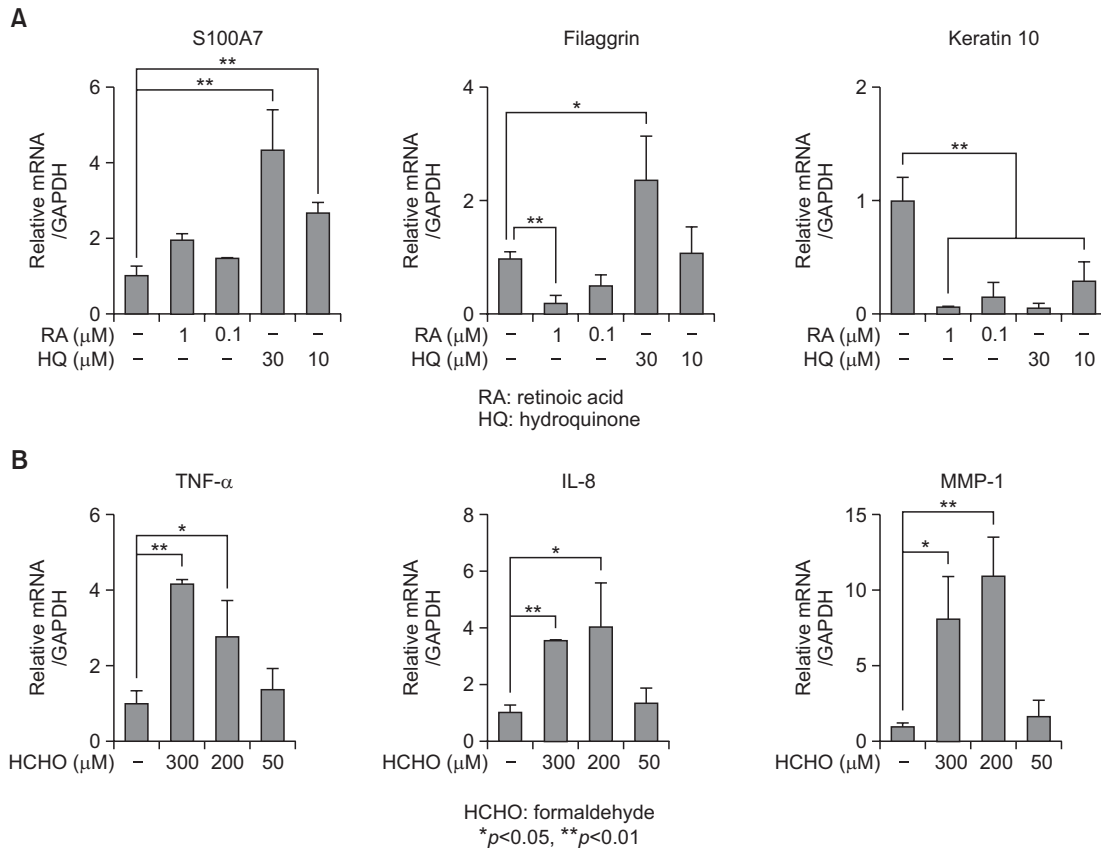
### Application of immortalized HFKs to cytotoxicity assessment

A HaCaT cell line, a spontaneously immortalized human keratinocyte, has been broadly used for skin research due to its unlimited passage competency. Thus, we wished to compare applicability of immortalized HFKs to cytotoxicity test in parallel with HaCaT. For this purpose, we first measured cytotoxicity of H<sub>2</sub>O<sub>2</sub> by using immortalized HFKs and HaCaT cells side by side. As shown in Fig. 5A, two to three fold difference was noticed in the ranges of CC<sub>50</sub> values obtained with these cell lines (629.0  $\mu$ M by HaCaT and 231.8  $\mu$ M by immortalized HFK7-E6E7) (Fig. 5A). When we extended our cytotoxicity assessment with a panel of eight compounds (2,4-dinitrochlorobenzene (#1), oxazolone (#2), benzylideneacetone (#4), 2,3-butanedione (#6), 1-butanol (#7), 6-methylcoumarin (#8), 4-methoxyacetophenone (#10), and octanoic acid (#19)), similar patterns of cytotoxicity profiles were also observed between two cell lines (Fig. 5B). 2,4-Dinitrochlorobenzene, which showed the most severe toxicity in the previous experiment prompted us to determine the more accurate CC<sub>50</sub> values for this compound by using HaCaT and immortalized HFK7-E6E7. As shown in Fig. 5C, CC<sub>50</sub> values obtained with these two cell lines turned out to be comparable to each other (4.5  $\mu$ M by HaCaT and 5.1  $\mu$ M by immortalized HFK7-E6E7). Therefore, we concluded that the immortalized HFKs can be equally applicable to toxicity assessment like HaCaT.

### Differentiation of immortalized HFKs

Most of suprabasal epidermal keratinocytes maintain different degree of differentiation. Therefore, efficient differentiation of HFKs is absolutely required to study differentiation-specific functions of skin. In order to compare differentiation capability of primary and immortalized HFKs, we incubated primary and immortalized HFKs in keratinocyte growth media (KGM) with 10% FBS and 1.8 mM calcium for four and seven days. Before differentiation, both primary and immortalized HFKs appear to stick to the culture plate trying to maintain single cell morphology and minimize their contact with neighboring cells (Fig. 6A). However, in the presence of high concentration FBS and calcium, both primary and immortalized HFKs started to conglomerate with each other trying to make mutually-connected colonies (Fig. 6B). This multiple colony-forming morphology was more evident in both primary and immortalized HFKs at the seventh day of differentiation (Fig. 6B). In order to study





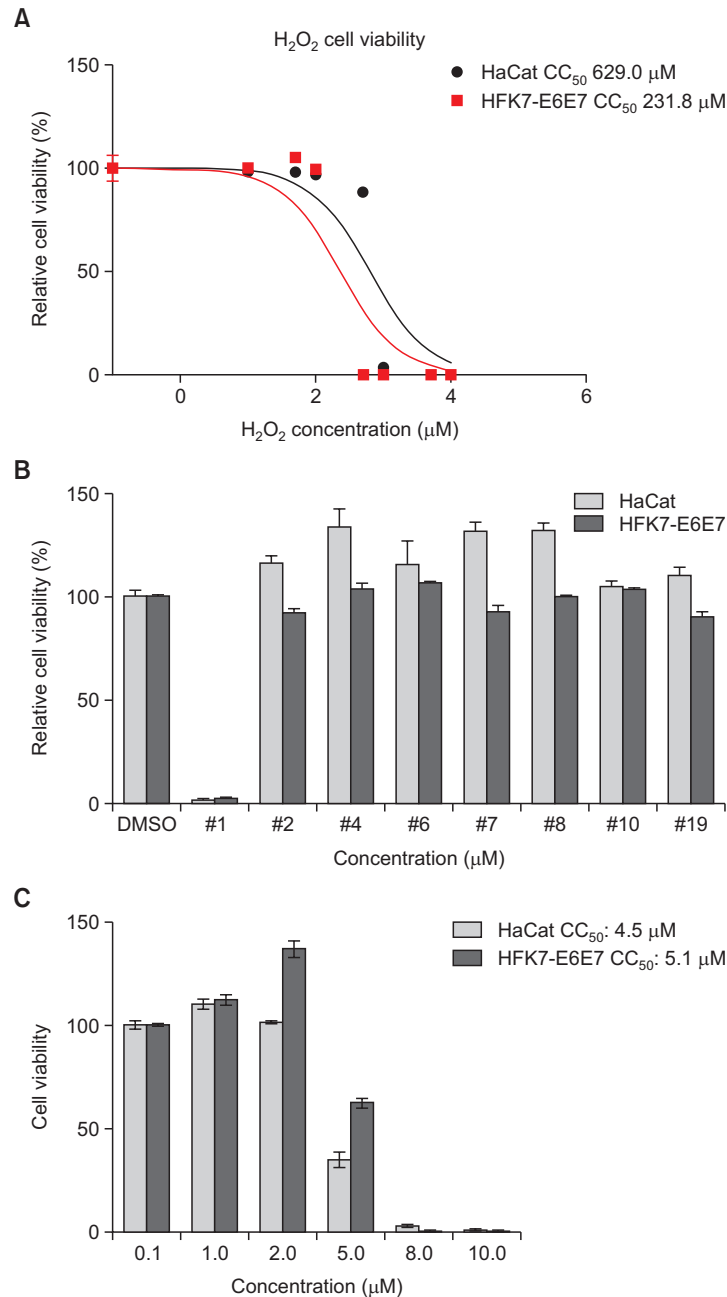
**Fig. 4.** Confirmation of responsiveness of immortalized HFK7-E6E7s cells to exogenous stimuli. Relative mRNA expression levels of differentiation marker genes such as S100A7, filaggrin, and keratin 10 in immortalized HFK7-E6E7s cells were measured by a real time RT-PCR analysis after treatment of (A) retinoic acid and hydroquinone (B) formaldehyde. Relative mRNA expression levels of pro-inflammatory genes such as TNF- $\alpha$ , IL-8, and MMP-1 in immortalized HFK7-E6E7s cells were measured by a real time RT-PCR analysis after treatment of formaldehyde.

their differentiation status in more accurate way, we decided to detect expression levels of epithelial differentiation marker proteins such as keratin 10 and involucrin by Western blot analysis. As shown in Fig. 6C, an early differentiation marker, keratin 10 was expressed only at the first and second day after differentiation. Its expression was completely lost in both primary and immortalized HFKs after three days of differentiation (Fig. 6C). However, both primary and immortalized HFKs were able to maintain continuous expression of a late differentiation marker, involucrin through the entire differentiation period (Fig. 6D). The results showed that the immortalized HFKs possess differentiation capability comparable to that of normal HFKs.

### Construction of 3D skin cultures by using immortalized HFKs

After confirming expression of epithelial differentiation markers in immortalized HFK in two-dimensional monolayer culture by using high FBS and calcium method, we wished to test applicability of HaCaT and three immortalized HFK lines (HFK4-E6E7, HFK6-E6E7, and HFK7-E6E7) to construction of two different kinds of 3D skin culture models. First, we subjected them to 3D skin culture system without a dermal equivalent (Fig. 7A). Air-liquid interface was introduced in this 3D culture system to induce full epithelial differentiation. Among three 3D cultures, those constructed by HaCaT and immor-

talized HFK7-E6E7 cells showed the best differentiation and stratification morphology with three-to-four cell thicknesses. However, those two 3D cultures made by immortalized HFK4-E6E7 and HFK6-E6E7 cells were only able to induce one or two cell depth-stratification (Fig. 7B). In order to verify expression of epithelial differentiation marker proteins, we conducted the immunohistochemical analysis of the 3D skin culture constructed by using immortalized HFK7-E6E7 cells. As shown in Fig. 7C, a keratinocyte marker, keratin 14 protein was diffusely expressed throughout the entire stratified epithelial tissues. In contrast, a cell proliferation marker, p63 showed restricted expression at basal layer (Fig. 7C). Moreover, filaggrin, a marker for more differentiated suprabasal layer, was rarely found. We also confirmed high expression of E-cadherin, a cell to cell junction marker in middle and upper parts of stratified area (Fig. 7C). After verifying limited differentiation in this dermis-free 3D culture system, we decided to try a dermal equivalent-containing 3D culture system (Fig. 8A). In this model, collagen matrix with mouse fibroblasts serves as the artificial dermis to support full differentiation of upper keratinocytes. In the 3D cultures raised by this technique, we were able to achieve a more complete stratification with much thicker differentiation (six to seven cells layer), which was comparable to that of actual patient foreskin sample (Fig. 8B, 8C). These results indicated that the immortalized HFKs are able to support the full



**Fig. 5.** Quantification of cell viability of HaCaT and immortalized HFK7-E6E7 cells in response to various reactive chemicals. (A) HaCaT and immortalized HFK7-E6E7 cells were treated with an increasing dose of H<sub>2</sub>O<sub>2</sub> for 72 hr and their relative cell viabilities were measured by MTT assay. (B) HaCaT and immortalized HFK7-E6E7 cells were incubated with a panel of eight compounds (2,4-dinitrochlorobenzene (#1), oxazolone (#2), benzylideneacetone (#4), 2,3-butanedione (#6), 1-butanol (#7), 6-methylcoumarin (#8), 4-methoxyacetophenone (#10), and octanoic acid (#19)) for 72 hr with a final concentration of 10 μM and their relative cell viabilities were measured by MTT assay. (C) HaCaT and immortalized HFK7-E6E7 cells were treated with an increasing dose of 2,4-dinitrochlorobenzene for 72 hr and their relative cell viabilities were measured by MTT assay.

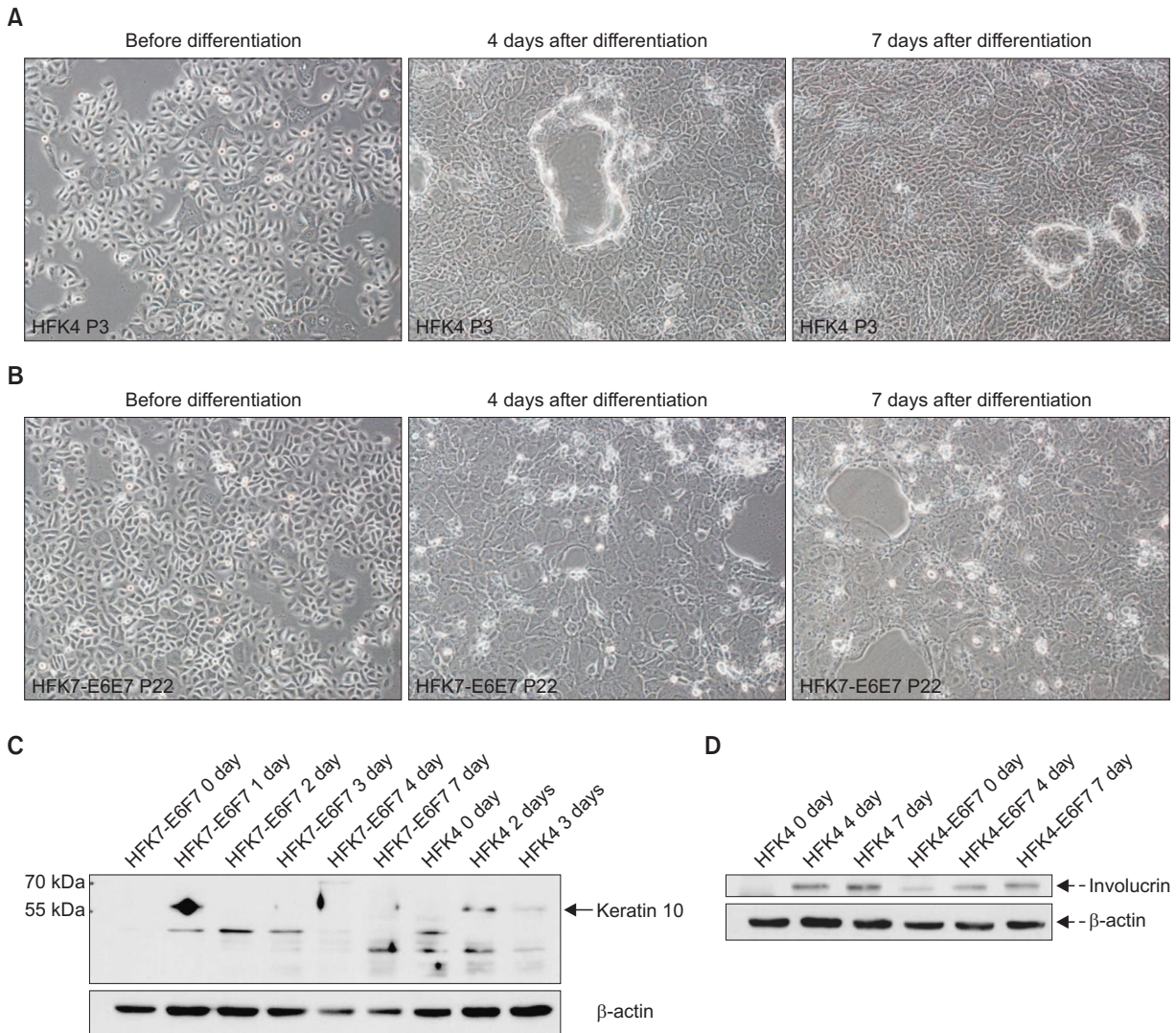
stratification and differentiation in the 3D skin cultures.

## DISCUSSION

In this report, we described successful isolation and establishment of immortalized HFKs by using primary HFKs through

a retroviral infection. Immortalization of HFKs was induced by inactivation of tumor suppressor genes, p53 and pRb through expression of high-risk HPV E6 and E7. In term of applicability to toxicity assessment and responsiveness to toxic chemicals, these newly established immortalized HFKs turned out to be comparable to HaCaT cells. Especially, differentiation capability of immortalized HFKs was much superior to HaCaT cells.





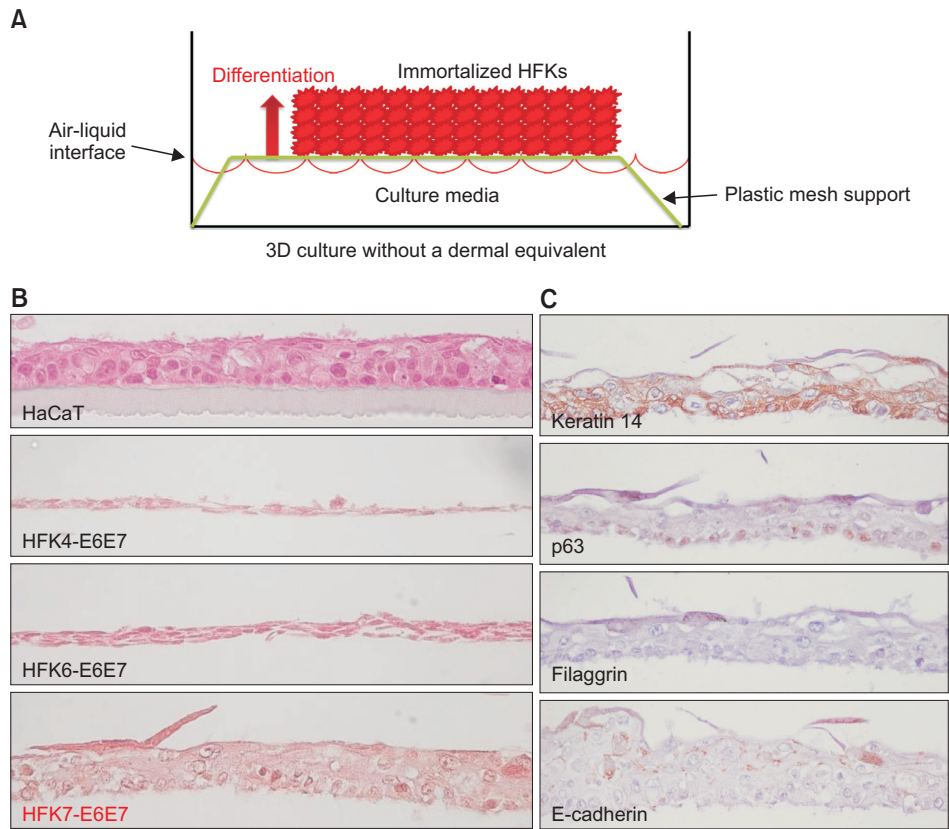
**Fig. 6.** Representative pictures of (A) primary and (B) immortalized HFKs incubated with high calcium and FBS (10% FBS) for 4 and 7 days for induction of epithelial differentiation. Two differentiation markers, (C) keratin 10 and (D) involucrin were detected in differentiated primary and immortalized HFKs by western blot assays.

Therefore, we hope immortalized HFKs will be a good replacement for HaCaT cell *in vitro* skin research.

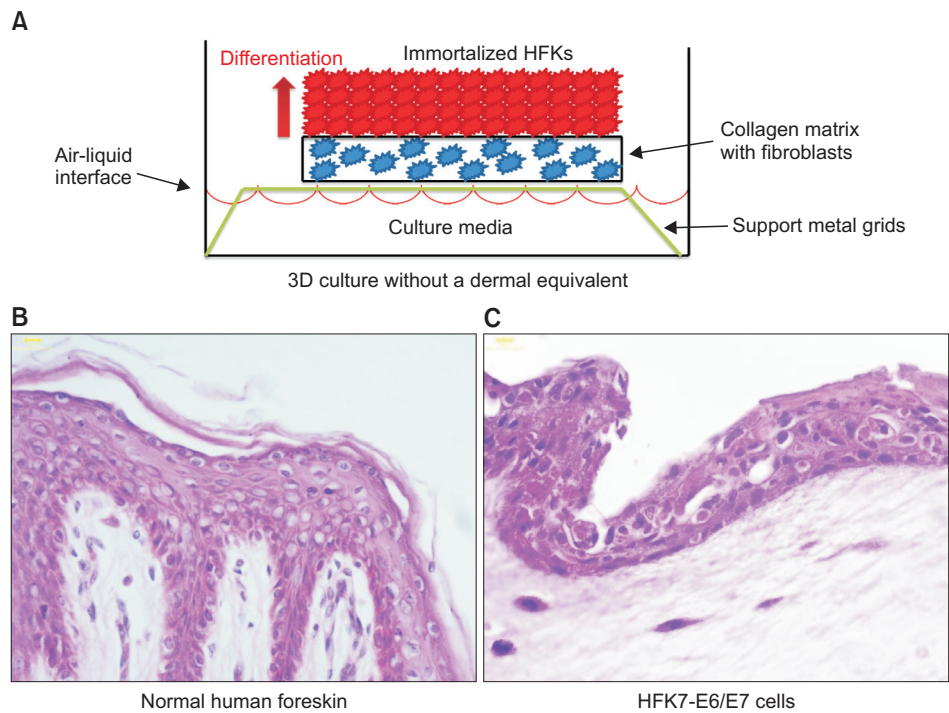
We were able to isolate seven HFKs lines from ten foreskin samples. Therefore, success rate for primary HFKs isolation was around 70% (Table 1). We were able to maintain this isolation success rate throughout four years of research period. Three commercially available HFK lines was not able to support the number of cells passages similar to that of patient-derived HFK lines probably due to previous cell passages by company before purchase. Since we were able to immortalize four HFKs lines out of eight HFKs lines, success rate for primary HFKs immortalization was around 50% (Table 2). Inconsistent titer of produced retroviruses might exert a negative effect on this rate for primary HFKs immortalization. Based on many experimental criteria including ease of handling, stable cell passage, maintenance of expression of marker proteins, immortalized HFK7-E6E7 cells were chosen as the best performer among four immortalized HFKs cell lines. How-

ever, due to technical difficulty for an unclear reason, we were not able to generate enough primary HFK7 cells. Therefore, we could not use them as a matching control in many experiments with immortalized HFK7-E6E7. Since primary HFK5 cells showed the most robust cell proliferation, they were used as a corresponding control to study expression of p53 and pRb in comparison with immortalized HFK5-E6E7s and an unmatched control in many other experiments (Fig. 3C).

Retinoic acid was shown to induce the keratinocyte differentiation by increasing expression of epidermal differentiation maker proteins such as S100A7, filaggrin, and keratin 10 (Cheong *et al.*, 2014). Based on this, we tried to test induction of these epidermal differentiation maker proteins in immortalized HFK7-E6E7 cells by treatment of 0.1 and 1 mM retinoic acid. As expected, we were able to see increased expression of S100A7 in immortalized HFKs by retinoic acid. However, retinoic acid failed to induce any change in expression levels of filaggrin and keratin 10 in immortalized HFKs (Fig. 4A). Fur-



**Fig. 7.** (A) A schematic diagram of 3D skin culture without a dermal equivalent. (B) 3D skin cultures with HaCaT, HFK4-E6E7, HFK6-E6E7, and HFK7-E6E7 cells were stained with hematoxylin and eosin after paraffin embedding. (C) Immunohistochemistry analysis of 3D skin cultures with HFK7-E6E7 to detect keratin 14, filaggrin, p63, and E-cadherin proteins.



**Fig. 8.** (A) A schematic diagram of 3D skin culture with a dermal equivalent. (B) Normal human foreskin and (C) 3D skin culture with HFK7-E6E7 cells were stained with hematoxylin and eosin after paraffin embedding.



ther study needs to be conducted to see if inability of retinoic acid to induce differentiation marker proteins is due to HPV E6/E7-induced immortalization or endogenous nature specific for HFK7 cells. Considering robust expression of pro-inflammatory cytokines in immortalized HFK7-E6E7 cells in response to stimulation by formaldehyde (Fig. 4B), we believe intact response mechanism is properly working in immortalized HFK7-E6E7 cells like normal primary HFKs.

In regards to differentiation capability, we confirmed the efficient expression of differentiation marker proteins such as keratin 10 and involucrin in immortalized HFK7-E6E7 cells upon induction of epidermal differentiation by high FBS and calcium (Fig. 6C, 6D). Early differentiation marker, keratin 10 was visible only when immortalized HFK7-E6E7 cells were incubated in high FBS and calcium media for 24 or 48 hrs. After this time point, expression level of keratin 10 returned to almost negligible level (Fig. 6C). In contrast to this observation, expression of involucrin was well maintained through entire differentiation period (Fig. 6C, 6D). We suspect that intermediate or late differentiation-specific nature of involucrin may affect this result.

In order to study the epithelial stratification capability of immortalized HFKs, we constructed two types of 3D skin cultures by using immortalized HFKs. The most prominent difference between these two systems is the absence or presence of a dermal equivalent. As shown in Fig. 7 and 8, much thicker and well-stratified 3D cultures could be constructed by using the 3D culture system with a dermal equivalent. These data indicate that mimicking the dermis by providing an extra basal layer, which was composed of mouse fibroblasts and collagen, plays a critical role in efficient differentiation and stratification of immortalized HFKs. Although we tried immunohistochemical analysis of 3D cultures with a dermal equivalent by using immortalized HFK7-E6E7, we were not able to draw any conclusion in regards to expression profiles of differentiation marker proteins due to poor quality of immunohistochemical analysis results. We are in the middle of improving quality of immunohistochemical analysis by trying different staining conditions.

When we tried to establish 3D skin culture by using HaCaT cells, we were not able to obtain well-differentiated 3D skin culture. This poor differentiation phenotype of HaCaT cells could not be improved by providing a dermal equivalent in 3D skin culture. This could be due to their endogenous lack of differentiation capability. In this regard, our newly established immortalized HFKs will be a better alternative to HaCaT cells when studying differentiation-dependent functions of skin. In addition, HFK cells were frequently used as HPV host cells due to its excellent *in vitro* differentiation capability, which is required for full maturation and particle formation of HPV (Lee and Laimins, 2004; Lee *et al.*, 2007). In regards to other example of immortalized HFK cell, McGhee *et al* reported HPV16-transformed foreskin keratinocyte cell line, 16-MT and used this cell line to analyze its molecular cytogenetic characteristics (McGhee *et al.*, 2006).

In conclusion, we successfully established immortalized HFKs by infecting isolated HFKs from patients with a retrovirus. They demonstrated unlimited cell passage, robust responsiveness to exogenous stimulation, comparable cytotoxicity profiles, and remarkable differentiation capability in both monolayer and 3D culture conditions. We believe that these newly generated HFK cell lines possess many features innate to normal skin, which are missing in HaCaT cell line. We hope

that broad distribution of these immortalized HFKs to skin biology researchers will facilitate animal-free *in vitro* skin study in the future.

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