

## Characterization of Keratinocytes, Fibroblasts and Melanocytes Isolated from Human Skin using Gene Markers

(Pencirian Keratinosit, Fibroblas dan Melanosit Dipencilkan daripada Kulit Manusia menggunakan Penanda Gen)

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### ABSTRACT

Cells isolated from skin have wide applications in studies of the pathogenesis of skin-related diseases and the construction of 3D skin equivalents. This study aimed to isolate keratinocytes, fibroblasts, and melanocytes from human foreskin and characterize the purity of the cell types. Keratinocytes, fibroblasts, and melanocytes from human foreskin were isolated by differential trypsinization and media selection. The purity of the cell types was characterized based on the expression of gene markers. The assessment of gene marker expression involved RNA extraction, primer design, quantitative polymerase chain reaction (qPCR) and immunocytochemical staining. Our results showed that in cocultures of keratinocytes and fibroblasts isolated from the dermis, fibroblasts could be separated from keratinocytes by quick trypsinization and culture in Dulbecco's modified Eagle's medium. The remaining keratinocytes are cultured in Epilife medium. Melanocytes in cocultures of melanocytes and keratinocytes isolated from the epidermis could be selected by changing Epilife medium to M254 medium. Gene marker results suggested that cytokeratin 14 (CK14) is a suitable marker for keratinocytes, elastin (ELN) is a suitable marker for fibroblasts, and tyrosinase (TYR) and tyrosinase-related proteins 1 and 2 (TYRP1 and TYRP2) are suitable markers for melanocytes. In conclusion, keratinocytes, fibroblasts, and melanocytes can be isolated from the same human foreskin sample by differential trypsinization and media selection techniques and characterized by suitable gene markers. This finding will aid in the isolation of pure skin cell types for various applications in regenerative medicine and toxicity studies.

Keywords: Epidermis; fibroblasts; gene markers; keratinocytes; melanocytes

### ABSTRAK

Sel yang dipencilkan daripada kulit mempunyai kegunaan meluas dalam kajian patogenesis penyakit berkaitan dengan kulit serta pembinaan setara kulit 3D. Kajian ini bertujuan untuk memencilkan sel keratinosit, fibroblas dan melanosit daripada kulit manusia serta mencirikan ketulenan jenis sel tersebut. Sel keratinosit, fibroblas dan melanosit telah dipencilkan dengan cara pentripsinan pembezaan dan pemilihan media. Sementara itu, ketulenan jenis sel telah dicirikan melalui ekspresi penanda gen. Pengekspresan penanda gen melibatkan pengekstrakan RNA, mereka bentuk primer, tindak balas rantai polimerase (qPCR) kuantitatif dan pewarnaan imunositokimia. Hasil yang diperoleh menunjukkan fibroblas di dalam ko-kultur sel keratinosit-fibroblas yang dipencilkan daripada lapisan dermis boleh diasingkan daripada keratinosit melalui proses pentripsinan pantas dan dikulturkan di dalam medium Eagle Pengubahsuaian Dulbecco, manakala sel keratinosit yang berbaki dikulturkan di dalam medium Epilife. Sel melanosit di dalam ko-kultur melanosit-keratinosit yang dipencilkan daripada lapisan epidermis boleh dipilih dengan menukarkan medium Epilife kepada medium M254. Keputusan penanda gen mencadangkan bahawa gen sitokeratin 14 (CK14) adalah penanda gen yang sesuai untuk sel keratinosit dan gen elastin (ELN) sesuai digunakan untuk sel fibroblas. Sementara itu, gen

tirosin (TYR) dan tirosinase berkaitan protein 1 dan 2 (TYRP1 dan TYRP2) sesuai digunakan untuk sel melanosit. Secara kesimpulannya sel keratinosit, fibroblas dan melanosit boleh dipencilkan daripada sumber kulit manusia yang sama melalui proses pentripsinan pembezaan dan teknik pemilihan media; serta dicirikan melalui penanda gen yang sesuai. Hasil kajian ini boleh membantu dalam memencilkan jenis sel kulit tulen untuk pelbagai kegunaan dalam perubahan regeneratif dan kajian kesitotoksikan.

Kata kunci: Epidermis; fibroblas; keratinosit; melanosit; penanda gen

## INTRODUCTION

Human skin consists of three layers, namely, the epidermis, dermis, and hypodermis (Aasen & Belmonte 2010). Keratinocytes and melanocytes can be isolated from the epidermis, whereas fibroblasts can be isolated from the dermis (Henrot et al. 2020). Cells isolated from the skin can be used to study the pathogenesis of skin-related diseases and as models for cosmetic toxicity tests. In addition, keratinocytes, fibroblasts, and melanocytes isolated from skin can be used to generate 3D skin equivalents (Gledhill et al. 2015). The isolation of cells from skin tissues involves the separation of epidermis from dermis using mechanical force or enzymatic digestion with dispase, thermolysin, and trypsin followed by dissociation of the tissue into a single cell suspension by proteolytic enzymes, such as collagenase and trypsin-EDTA (Henrot et al. 2020; Hybbinette et al. 1999). In addition, various culturing protocols for isolated cells have been reported; for example, isolated keratinocytes can be cultured on feeder cells (Rheinwatd & Green 1975) or in serum-free defined media (Tenchini et al. 1992). Defined media have the advantage of avoiding feeder cell contamination, but cell proliferation is slower in defined media compared with serum- or feeder-based media.

A common problem associated with cell isolation is the contamination of other cell types from skin tissues (Szymański et al. 2020). For example, keratinocytes, and fibroblasts are typically isolated together from the dermis, whereas keratinocytes and melanocytes are isolated together from the epidermis. A combination of differential trypsinization and media selection techniques could be used to isolate keratinocytes, fibroblasts, and melanocytes from the skin based on their differential adhesive strength in culture flasks and nutrient requirements (Caneparo et al. 2020). In the coculture of keratinocytes and fibroblasts isolated from the dermis, a quick trypsin-EDTA incubation could dissociate the fibroblasts from the coculture while leaving the keratinocytes attached to the culture flask because keratinocytes attached more strongly than fibroblasts to

the flask (Zuliani et al. 2013). The separated cell types could be cultured in specific media that promote their growth, namely, Epilife medium with growth supplements for keratinocytes and DMEM/FBS for fibroblasts. In the coculture of keratinocytes and melanocytes isolated from the epidermis, the use of melanocyte medium, such as M254 with appropriate growth supplements, could promote the growth of melanocytes while suppressing keratinocytes proliferation.

Several genes have been reported to be useful for cell type identification based on their function in that particular cell type. Cytokeratin 14 (*CK14*) is expressed in dividing basal keratinocytes and is involved in maintaining the shape of epidermal cells and resisting mechanical stress (Byrne et al. 1994). Cytokeratin 19 (*CK19*) is expressed in basal keratinocytes and has been suggested as a marker for epidermal homeostasis and as an indicator of young growing skin (Pontiggia et al. 2009). E-cadherin (*CDH1*) is a calcium-dependent transmembrane glycoprotein that is involved in keratinocyte intercellular adhesion and stratification (Charest et al. 2009; Hines et al. 1999). Elastin (*ELN*) is a major fibrillary protein component of elastic fibres produced by fibroblasts (Giro et al. 1985). Fibronectin (*FNI*) and collagen (*COL1A1*) are components of the wound healing extracellular matrix produced by fibroblasts (Werner & Grose 2003). Melanocytes are characterized by melanocyte-specific markers, such as tyrosinase (*TYR*), tyrosinase-related protein 1 (*TYRP1*) and tyrosinase-related protein 2 (*TYRP2*) activities (Cichorek et al. 2013). Hence, these markers could be used to characterize the purity of isolated cells from the skin. Accurate markers are important for the isolation of pure cell types for various applications, i.e. for the study of skin-related diseases and models used for cosmetic toxicity tests, in addition to other applications in regenerative medicine. This study aimed to isolate and culture keratinocytes, fibroblasts and melanocytes from the same human foreskin sample using differential trypsinization and media selection techniques and to

characterize the purity of the cell types based on their respective gene markers.

## MATERIALS AND METHODS

### MEDIA PREPARATION

Epilife medium (Gibco, Carlsbad, CA, USA) consisted of 60  $\mu$ M calcium chloride with added human keratinocyte growth supplement (Gibco), 1% penicillin/streptomycin (HyClone, Logan, UT, USA) and 1% amphotericin B (PAA Austria). Complete culture medium (CCM) was composed of DMDM (HyClone) with 10% FBS (PAA). M254 (Gibco) was added to human melanocyte growth supplement-2 (Gibco), 1% penicillin/streptomycin and 1% amphotericin B.

### CELL ISOLATION AND CULTURE

This project was approved by the Ethics and Research Committee of Universiti Kebangsaan Malaysia with approval code FF-355-2010. Human foreskin samples ( $n=6$ ) of children between 9-12 years old were collected and processed after obtaining consent from their parents. First, the foreskin sample was washed with 70% ethanol. The sample was rinsed with phosphate-buffered saline (PBS) containing 10% amphotericin B, 10% penicillin/streptomycin and 1% gentamycin (PAA). The foreskin was transferred into a petri dish and cut into small pieces. The dermis was then separated from the epidermis. The skin sample was digested with 0.3% type I collagenase (Worthington, Lake Wood USA) in a shaker incubator at 37 °C for 3 h. Then, the dermis of the skin was digested with the epidermis layer remaining in suspension. The dermis fibroblasts and epidermis were pelleted down by centrifugation at 500  $\times$  g for 10 min. The supernatant was discarded, and 10 mL of 0.05% trypsin-EDTA (TE, Invitrogen) was added to the epidermis to dissociate the keratinocytes in the epidermis. The sample was agitated at 37 °C in a water bath for 10 min. Then, 10 mL of defined trypsin inhibitor (DTI, Invitrogen) was added to inactivate the TE reaction. The sample was centrifuged at 750  $\times$  g for 10 min to collect the cells and nondigested epidermis. The supernatant was discarded, and the pellet was suspended in PBS (the epidermis layers were carefully removed from the cell suspension and placed into a new tube). The cell suspension containing mainly fibroblasts and keratinocytes was centrifuged at 625  $\times$  g for 10 min to pellet the cells. Then, the cells were resuspended in Epilife:CCM at a ratio of 1:1 to culture the fibroblasts and keratinocytes in a coculture

system and seeded in a 6-well plate (NUNC, Roskilde, Denmark). Culture medium was changed every 2-3 days. The morphology of the cells in the coculture was observed under an inverted light microscope daily.

The tube containing nondigested epidermis layers was processed again with 10 mL of 0.05% TE in a 37 °C water bath for 10 min. Then, 10 mL of DTI was added to inactivate the TE, and the cells were pelleted by centrifugation at 750  $\times$  g for 10 min. The supernatant was discarded, and the cell pellet containing melanocytes and keratinocytes was resuspended in Epilife medium and seeded in a 6-well plate.

### SELECTION OF FIBROBLASTS AND KERATINOCYTES

After 6-7 days in culture, the cells in the coculture of fibroblasts and keratinocytes at passage 0 (P0-f/k) reached 60-70% confluency and were ready for trypsinization to remove the dermal fibroblasts. Quick trypsinization removed the fibroblasts while leaving the keratinocytes to grow in clusters. Briefly, the culture medium was discarded, and the well was rinsed with 2 mL of PBS to wash the cells. Then, 2 mL of 0.05% TE was added to the wells and incubated in a 5% CO<sub>2</sub> incubator at 37 °C for 5 min. Two millilitres of DTI were added to stop the TE reaction, and the cell suspension containing dermal fibroblasts was transferred into a new tube. The well was rinsed with PBS, and 2 mL of Epilife medium was added to the well to culture the remaining keratinocytes at passage 0 (P0-k). When the keratinocytes were confluent, the cells were trypsinized and subcultured to become passage 1 (P1-k). The removed fibroblasts were then cultured in T75 flasks with CCM and labelled passage 1 (P1-f).

### SELECTION OF MELANOCYTE CULTURES

After 3 days of melanocyte-keratinocyte coculture at passage 0 (P0-m/k), the Epilife medium was changed to melanocyte-specific medium M254 (Gibco). M254 medium supported melanocyte growth but not keratinocyte growth. After the culture was maintained in M254 medium for 7 days, melanocytes with dendritic features started to proliferate exponentially. The melanocyte culture was subcultured in M254 medium until confluent, and the next passage was labelled P1-m.

### RNA EXTRACTION

Total RNA of cultured cells (coculture, keratinocytes, fibroblasts, and melanocytes) was extracted using TRI reagent (Molecular Research Center, Cincinnati, OH,

USA) according to the manufacturer's instructions. Polyacryl carrier (Molecular Research Center) was added to precipitate the total RNA. The extracted RNA pellet was resuspended in RNase/DNase-free water (Invitrogen, USA). RNA samples were stored at -80 °C immediately after extraction.

#### PRIMER DESIGN

Primers for human *GAPDH*, *CK14*, *CK19*, *CDH1*, *ELN*, *FNI*, *COL1A1*, *TYR*, *TYRP1*, and *TYRP2* were designed with Primer 3 Output software (Koressaar & Remm 2007) and blasted with GenBank database sequences (Ncbi 1988). The sequences of the primers are shown in Table 1.

QUANTITATIVE POLYMERASE CHAIN REACTION (qPCR) mRNA expression of selected gene markers was quantitated by reverse transcription qPCR (RT-qPCR). RT-qPCR was performed using iScript One-step RT-PCR kits with SYBR Green (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendation. The thermal cycler was set as follows: 50 °C for 20 min, 94 °C for 5 min, and 38 cycles of 95 °C for 10 s and 61 °C for 30 s. After PCR amplification, melting curve analysis was performed to determine the specificity of the PCR product. Gene expression levels were normalized to *GAPDH*.

#### IMMUNOCYTOCHEMICAL STAINING

Gene markers that were specific for specific cell types as shown by qPCR were selected for validation at the protein level by immunocytochemical staining (in this case: CK14, Tyr and Elastin). Cells were cultured in Teflon-coated slides and fixed in 4% paraformaldehyde when they reached confluence. A DAKO REAL EnVision detection system kit (Glostrup, Denmark) was used for immunostaining. Antigen retrieval was performed for anti-CK14 and anti-elastin staining using sodium citrate buffer pH 6.0, whereas antigen retrieval for anti-Tyr was performed using Tris-EDTA pH 9.0. Anti-CK14 antibody (LL002; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted at 1:500 with a 30-min incubation, anti-Tyr (T311; DAKO) was diluted at 1:1000 a 30-min incubation, and anti-Elastin (BA-4; Santa Cruz Biotechnology) was diluted at 1:200 for a 1-h incubation.

#### STATISTICAL ANALYSIS

Data are reported as the mean  $\pm$  standard error of mean (SEM) and the number of samples,  $n = 6$ . Student's t-test

was used when the data were normally distributed, whereas the nonparametric test was performed when the data were not normally distributed.  $P < 0.05$  was considered statistically significant.

## RESULTS AND DISCUSSION

#### MORPHOLOGY OF THE CULTURED CELLS

Keratinocytes, fibroblasts, and melanocytes have different morphological features that can be easily differentiated under a light microscope. Keratinocytes are round or polygonal in shape and grow in clusters, fibroblasts have spindle shapes, and melanocytes are stellar in shape with dendrites. After 5-6 days (P0-k/f) of keratinocyte-fibroblast coculture in Epilife:CCM (1:1), a mixture of cells with polygonal, spindle or dendritic shapes was noted (Figure 1(A)). After quick trypsinization at passage 0, the cells were changed to Epilife medium and cultured for 4-5 days (P0-k). The majority of the cells had a round morphology, whereas some cells had a spindle shape (Figure 1(B)). At passage 1 (P1-k), most cells remained round in shape (Figure 1(C)). The cells collected from quick trypsinization were grown in CCM, and the cells (P1-f) had a spindle-like shape (Figure 1(D)).

In the coculture of melanocytes and keratinocytes, some of the cells exhibited small dendrites in Epilife medium on the first day (Figure 1(E)) and became more dendritic when the medium was changed to M254 on Day 3 (Figure 1(F)). When the coculture was confluent (P0-m/k), the majority of the cells appeared star-like in shape with some cells having round morphology (Figure 1(G)). After trypsinization and culture until confluent (P1-m), most of the cells appeared stellar in shape with dendrites (Figure 1(H)).

In this study, based on morphological observation after differential trypsinization and media selection, P0-k/f culture likely consisted of keratinocytes, fibroblasts, and melanocytes; P0-k consisted of keratinocytes and fibroblasts; P1-k consisted of keratinocytes; P1-f consisted of fibroblasts; P0-m consisted of melanocytes and keratinocytes; and P1-m consisted of melanocytes. However, cell purity must be characterized based on gene markers, and suitable markers need to be selected for particular cell types.

#### GENE MARKER ANALYSIS AND

#### IMMUNOCYTOCHEMICAL STAINING OF GENE MARKERS

*CK14* was highly expressed in P1-k cells followed by P0-k and P0-k/f cells (Figure 2(A)). Its expression was

very low but detectable in P0-m/k and P1-f cells. *CK19* was highly expressed in P0-k/f cells followed by P0-k, P1-f and P1-k cells (Figure 2(B)). Its expression was very low but detectable in P0-m/k cells. *CDH1* was highly expressed in P1-k cells followed by P0-k, P0-k/f and P1-m cells (Figure 2C). Its expression was very low but detectable in P0-m/k and P1-f cells.

The highest levels of *ELN* expression were noted in P1-f cells followed by P0-k/f cells (Figure 3(A)). Its expression was very low but detectable in P0-k cells. *FNI* was highly expressed in P1-m cells followed by P1-f, P0-m/k, P0-k/f and P0-k cells (Figure 3(B)). Its expression was very low but detectable in P1-k cells. *COL1A1* was highly expressed in P1-f cells followed by P0-k/f, P0-k, P0-m/k and P1-m cells (Figure 3(C)). Its expression was very low but detectable in P1-k cells.

Moreover, *TYR* was highly expressed in P0-m/k cells followed by P1-m, P0-k/f and P0-k cells (Figure 4(A)). Its expression was very low but detectable in P1-f cells. *TYRP1* expression was the highest in P1-m cells followed by P0-m/k, P0-k/f and P0-k cells (Figure 4(B)). Its expression was very low but detectable in P1-k and P1-f cells. *TYRP2* was highly expressed in P0-m/k cells followed by P1-m and P0-k/f cells (Figure 4(C)). Its expression was very low but detectable in P0-k and P1-f cells.

Based on the gene expression results, immunocytochemical staining was performed to verify the presence of specific proteins in different types of cells. Keratinocytes were positively stained for anti-CK14 (Figure 5(A), 5(B) and 5(C)), fibroblasts were positively stained for anti-elastin (Figure 5(D)) and melanocytes were positively stained for anti-Tyr (Figure 5(E) and 5(F)).

Based on these results, *CK14* is a suitable marker for keratinocytes because it was highly expressed in P1-k cells, which is consistent with previous studies reporting the expression of *CK14* in keratinocytes (Borowiec et al. 2013; Velez-delValle et al. 2016). High *CK14* expression levels in P1-k culture indicate that the cultured cells were in their mitotically active phase (Fuchs & Green 1980). The presence of *CK14* expression in the P0-k/f and P0-k cells indicated that these cell cultures contained keratinocytes. The absence of *CK14* expression in P1-m indicated that it is a good marker for keratinocytes given that the gene was not expressed in melanocytes. Very low *CK14* expression in P1-f cells suggested that a very low number of keratinocytes was present in the culture.

However, *CK19* is not a suitable marker for keratinocytes as it was expressed in both P1-k and P1-f

cells, suggesting that it cannot be used to differentiate between keratinocytes and fibroblasts. *CDH1* was also not suitable as a marker for keratinocytes as it was expressed in both P1-k and P1-m cells. A previous study showed that E-cadherin found in melanocytes was involved in melanocyte-keratinocyte adhesion; therefore, *CDH1* expression was observed in melanocytes (Tang et al. 1994).

*ELN* is a suitable marker for fibroblasts given that it was highly expressed in P1-f cells but absent in P1-k and P1-m cells. *FNI* is not a suitable marker for fibroblasts because it was expressed in both P1-f and P1-m cells. It remains unclear why *FNI* is highly expressed in melanocytes. Although *COL1A1* was highly expressed in P1-f cells, it is not suitable as a marker for fibroblasts because it was also expressed in P1-m cells.

Furthermore, *TYR*, *TYRP1*, and *TYRP2* are considered suitable markers for melanocytes because they were expressed in P1-m cells but absent or present at very low levels in P1-k and P1-f cells. However, *TYR* and *TYRP2* expression was much higher in P0-m cells compared with P1-m cells. A previous study showed that miRNA in exosomes of keratinocytes stimulated the activity of tyrosinase in melanocytes (Cicero et al. 2015). In this study, high *TYR* expression in P0-m culture may be due to the presence of keratinocytes in the coculture that stimulated *TYR* expression in melanocytes. However, it is unclear whether *TYRP2* expression is also stimulated by *TYR*.

Consistent with the morphological observation, P0-k/f cultures, which consist of cells with round, spindle and stellar shapes, expressed *CK14*, *ELN*, *TYR*, *TYRP1*, and *TYRP2*, suggesting that this culture consists of keratinocytes, fibroblasts, and melanocytes. In contrast, P0-k culture with round and spindle cells expressed *CK14*, *TYR*, and *TYR1* and very low levels of *ELN* and *TYRP2*, suggesting the presence of keratinocytes, melanocytes and fibroblasts. The fibroblast number, which corresponds with *ELN* expression in the P0-k culture, may be too low. In addition, melanocytes are present in the P0-k culture but may not be observed by visible examination. The P0-m culture consisted of stellar and round cells, suggesting that these cells were melanocytes and keratinocytes, respectively, which was consistent with the gene marker results showing the expression of *TYR*, *TYRP1*, and *TYRP2* with very low *CK14* expression.

In summary, isolation of pure primary cells, such as keratinocytes, fibroblasts, and melanocytes, from the skin is essential in the field of translational research, especially for skin diseases with a complex

pathophysiology where different cell types may be simultaneously affected (Henrot et al. 2020). Cells isolated from healthy subjects or patient samples can be

further used in 3D skin models to better reproduce the complex pathophysiology of the disease and provide a model for product testing or toxicity studies.

TABLE 1. Primer sequences, PCR product size and accession number for gene expression analysis

Genes	Accession numbers	Primer sequences	PCR product size (bp)
<i>TYRP2</i>	NM_001922.2	F: 5'-AGATTGCCTGTCTCTCCAGAAG-3' R: 5'-CTTGAGAATCCAGAGTCCCATC-3'	116
<i>TYRP1</i>	NM_000550.1	F: 5'-GCTCCAGACAACCTGGGAT-3' R: 5'-TCAGTGAGGAGAGGCTGGTT-3'	185
<i>TYR</i>	NM_000372.3	F: 5'-GATGAGTACATGGGAGGTCAGC-3' R: 5'-GTACTCCTCCAATCGGCTACAG-3'	102
<i>COL1A1</i>	NM_000088	F: 5'-AGGGCTCCAACGAGATCGAGATCCG-3' R: 5'-TACAGGAAGCAGACAGGGCCAACG-3'	222
<i>FNI</i>	NM_002026.2	F: 5'-CGGTTGTTATGACAATGGAAAA-3' R: 5'-TCGGCTTCCTCCATAACAAG-3'	100
<i>ELN</i>	NM_000501	F: 5'-GGCCTGGAGGCAAACCTCTT-3' R: 5'-CCACCAACTCCTGGGACACC-3'	189
<i>CK19</i>	NM_002276.4	F: 5'-TTTGAGACGGAACAGGCTCT-3' R: 5'-AATCCACCTCCACACTGACC-3'	211
<i>CK14</i>	NM_000526.4	F: 5'-GGCCTGTCTGTCTCATCCTC-3' R: 5'-GAGGTTCTGCATGGTCACCT-3'	222
<i>CDH1</i>	NM_004360.3	F: 5'-TGCCAGAAAATGAAAAAGG-3' R: 5'-GTGTATGTGGCAATGCGTTC-3'	200
<i>GAPDH</i>	NM_002046	F: 5'-TCCCTGAGCTGAACGGGAAG-3' R: 5'-GGAGGAGTGGGTGTCGCTGT-3'	217

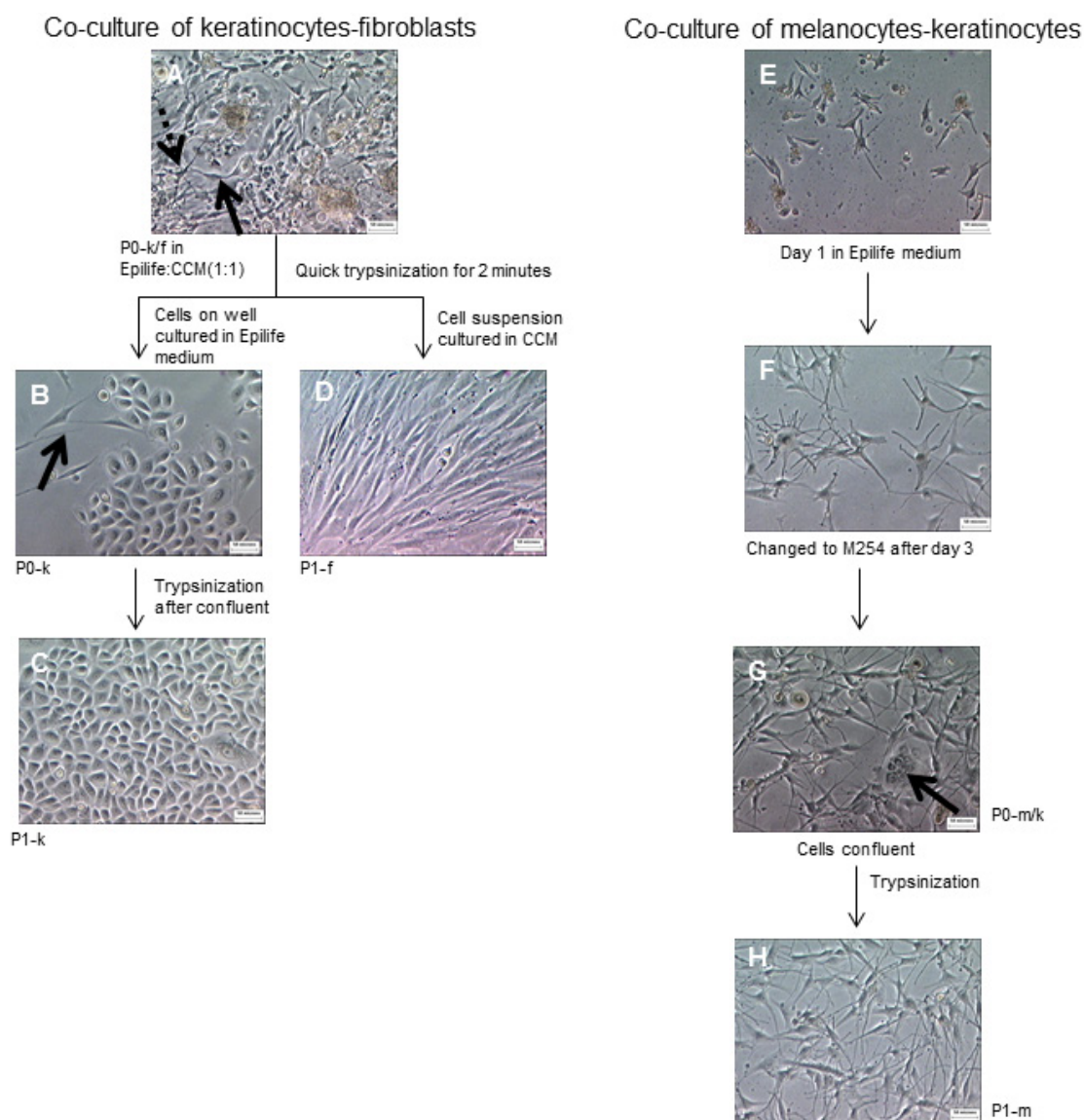


FIGURE 1. Morphology of cocultures of keratinocytes and fibroblasts as well as melanocytes and keratinocytes. (A) Coculture of keratinocytes and fibroblasts in Epilife medium:CCM (1:1) after 5-6 days. The majority of the cells had round shapes, whereas some cells had spindle (solid arrow) or stellar shapes (line arrow), (B) After quick trypsinization for 2 min and culture in Epilife medium for 4-5 days, most of the cells appeared round, whereas some cells appeared spindle (arrow), (C) At passage 1, most cells appeared round in shape, (D) Cells collected from quick trypsinization at passage 0 were grown in CCM, and most cells were spindle in shape, (E) Coculture of melanocytes and keratinocytes in Epilife medium on Day 1, (F) The culture medium was changed to M254 on Day 3, (G) When confluent, the majority of the cells exhibited a stellar shape, whereas some cells were round in shape (arrow), and (H) At passage 1, most of the cells appeared stellar in shape. Representative photomicrograph of  $n = 6$ . Scale bar: 50  $\mu$ M

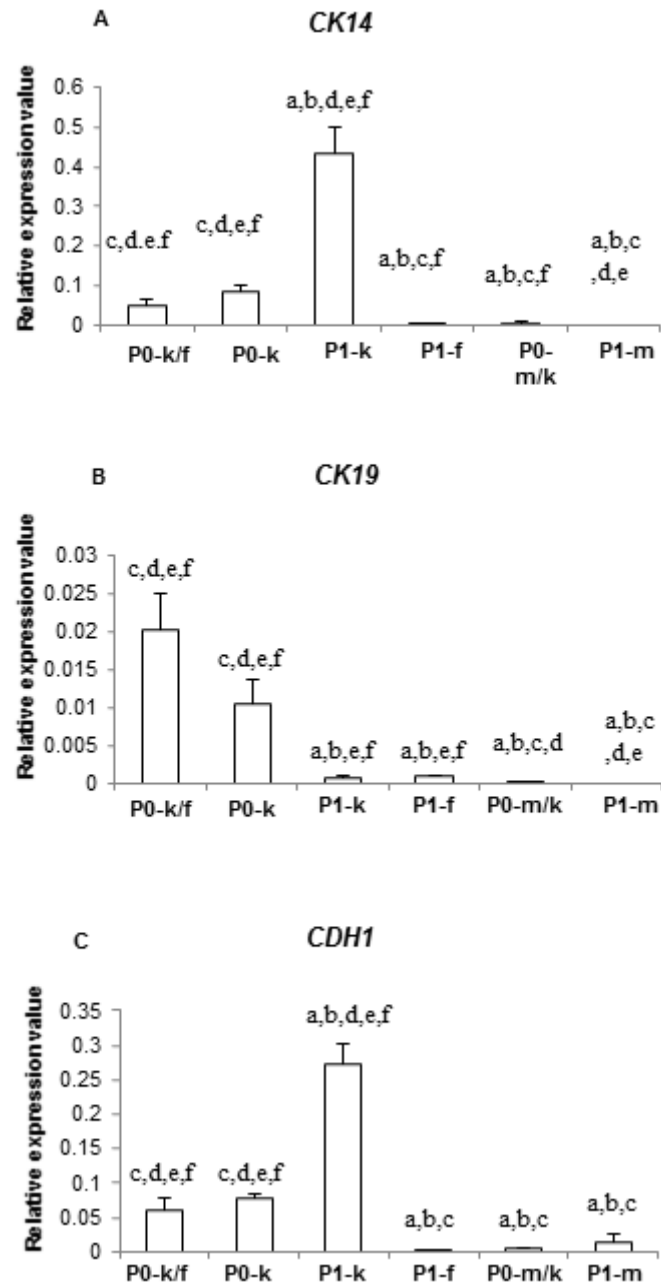


FIGURE 2. Relative gene expression levels of (A) CK14, (B) CK19 and (C) CDH1 in different cell cultures. P0-k/f: coculture of keratinocytes and fibroblasts at passage 0 in Epilife:CCM (1:1); P0-k: culture of keratinocytes after quick trypsinization and change to Epilife medium at passage 0; P1-k: culture of keratinocytes at passage 1; P1-f: culture of fibroblasts in CCM after collection from quick trypsinization; P0-m/k: coculture of melanocytes and keratinocytes in M254 at passage 0; P1-m: culture of melanocytes at passage 1. <sup>a</sup> $p < 0.05$  compared to P0-k/f; <sup>b</sup> $p < 0.05$  compared to P0-k; <sup>c</sup> $p < 0.05$  compared to P1-k; <sup>d</sup> $p < 0.05$  compared with P1-f; <sup>e</sup> $p < 0.05$  compared to P0-m/k; <sup>f</sup> $p < 0.05$  compared to P1-m. Value was expressed in mean $\pm$ SEM,  $n = 6$



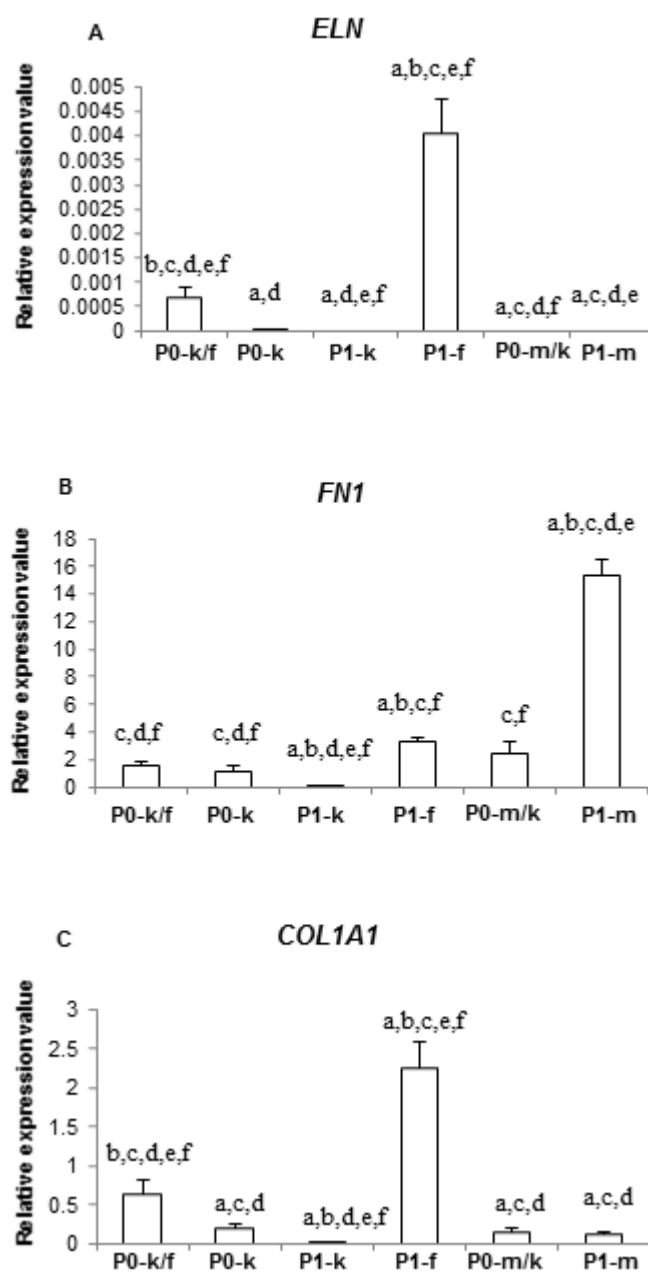


FIGURE 3. Relative gene expression levels of (A) *ELN*, (B) *FN1*, and (C) *COL1A1* in different cell cultures. P0-k/f: coculture of keratinocytes and fibroblasts at passage 0 in Epilife:CCM (1:1); P0-k: culture of keratinocytes after quick trypsinization and change to Epilife medium at passage 0; P1-k: culture of keratinocytes at passage 1; P1-f: culture of fibroblasts in CCM after collection from quick trypsinization; P0-m/k: coculture of melanocytes and keratinocytes in M254 at passage 0; P1-m: culture of melanocytes at passage 1. <sup>a</sup> $p < 0.05$  compared to P0-k/f; <sup>b</sup> $p < 0.05$  compared to P0-k; <sup>c</sup> $p < 0.05$  compared to P1-k; <sup>d</sup> $p < 0.05$  compared with P1-f; <sup>e</sup> $p < 0.05$  compared to P0-m/k; <sup>f</sup> $p < 0.05$  compared to P1-m. Value is expressed in mean $\pm$ SEM,  $n = 6$

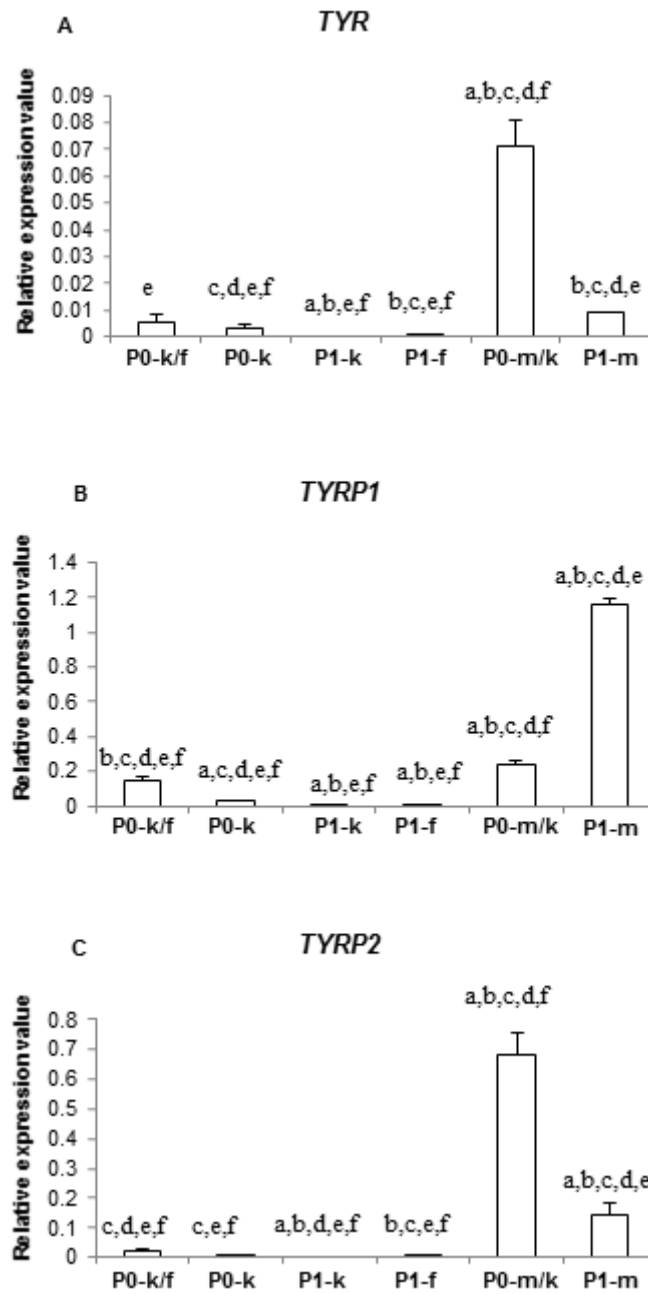


FIGURE 4. Relative gene expression levels of (A) *TYR*, (B) *TYRP1* and (C) *TYRP2* in different cell cultures. P0-k/f: coculture of keratinocytes and fibroblasts at passage 0 in Epilife:CCM (1:1); P0-k: culture of keratinocytes after quick trypsinization and change to Epilife medium at passage 0; P1-k: culture of keratinocytes at passage 1; P1-f: culture of fibroblasts in CCM after collection from quick trypsinization; P0-m/k: coculture of melanocytes and keratinocytes in M254 at passage 0; P1-m: culture of melanocytes at passage 1. <sup>a</sup> $p < 0.05$  compared to P0-k/f; <sup>b</sup> $p < 0.05$  compared to P0-k; <sup>c</sup> $p < 0.05$  compared to P1-k; <sup>d</sup> $p < 0.05$  compared with P1-f; <sup>e</sup> $p < 0.05$  compared to P0-m/k; <sup>f</sup> $p < 0.05$  compared to P1-m. Value was expressed in mean $\pm$ SEM,  $n = 6$

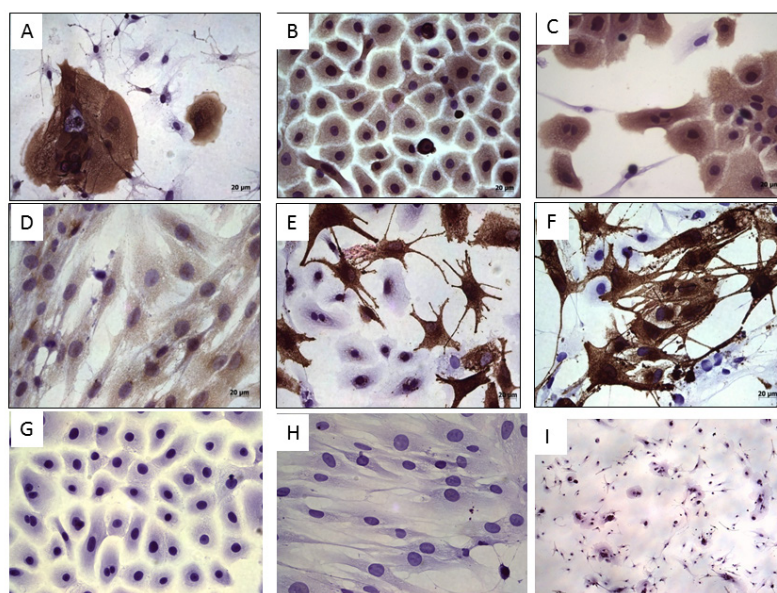


FIGURE 5. Immunocytochemical staining. (A) Anti-CK14 (1:500) only stained keratinocytes in the culture mixed with other cells, (B) Positive anti-CK14 staining in the homogeneous culture of keratinocytes (P1 keratinocytes), (C) Specific positive anti-CK14 staining on keratinocytes in the culture that contained some fibroblasts, (D) Positive anti-elastin (1:200) staining of the cultured fibroblasts, (E) and (F) Specific positive anti-Tyr (1:1000) staining on melanocytes (P1 melanocytes) in the mixed culture with keratinocytes. Negative cells stained appear blue (G, H, I) (X400)

#### CONCLUSION

Keratinocytes, fibroblasts, and melanocytes could be isolated from the same human foreskin using differential trypsinization and media selection techniques. *CK14* and *ELN* represent suitable markers for keratinocytes and fibroblasts, respectively, and *TYR*, *TYRP1*, and *TYRP2* are suitable markers for melanocytes.

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