·Basic Research·

In vitro transdifferentiation of corneal epithelial-like cells from human skin-derived precursor cells

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Abstract

• The damage of human corneal cells encounter with the problem of availability of corneal cells for replacement. Limitation of the source of corneal cells has been realized. An attempt of development of corneal epithelial-like cells from the human skin-derived precursor (hSKPs) has been made in this study. Combination of three essential growth factors: epidermal growth factor (EGF), keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) could demonstrate successfully induction of hSKPs to differentiation into corneal cells.

The induced cells expressed the appearance of markers of corneal epithelial cells as shown by the presence of keratin 3 (K3) by antibody label and Western blot assay. The K3 gene expression of induced hSKPs cells as shown by reverse transcription-polymerase chain reaction (RT-PCR) technology was also demonstrated. The presence of these markers at both gene and protein levels could lead to our conclusion that the directional transdifferentiation of hSKPs cells into corneal epithelial cells was successfully done under this cell induction protocol. The finding shows a newly available stem cell source can be obtained from easily available skin. Cells from autologous human skin might be used for corneal disorder treatment in future clinical application.

• KEYWORDS: corneal epithelial-like cell; human skin-derived precursor cell; transdifferentiation

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INTRODUCTION

Ocular surface is easily accessible to external exposure. The ocular surface disorders may be found in both acquired and inherited disorders, such as chemical injury, physical injury, and Stevens-Johnson syndrome. Although, cells in corneal epithelium are highly regenerative, they are always prone to possible injuries. These injuries are leading to corneal epithelium inflammation and neovascularization from conjunctival epithelium. Upon aggressive damage as limbal stem cell deficiency (LSD), the migration of conjunctival epithelium are leading to loss of visual acuity and uncovering treatment by above methods[1].

Limbal stem cells (LSC) are located at the basal layers of a limbus, which is a transitional zone between cornea and sclera. They are showing the potential of stem cells for the LSD treatment which called LSC transplantation or the *ex vivo* cultivated LSC transplantation. The procedure of this method is isolated putative LSC from limbal biopsies, expanded by the culture system, seeded onto a scaffold (such as the amniotic membrane or synthetic membrane) and transplanted to the recipients. This method has been reported in both animals[2,3] and human models[4-6] for LSD treatment. The histocompatibility between donor LSC and the recipient, the risk of tissue injury from harvested biopsies and not useful for treating bilaterally LSD is a limitation of this method. Other stem cell sources such as oral mucosal epithelium cells [7-10], mesenchymal stem cells [11], embryonic stem cells[12,13], neural crest-derived stem cell-like cells[14] and immature dental pulp stem cells[15] are established for corneal epithelium replacement, but until now no long-term results have been reported.

Our previous finding demonstrated that human skin-derived precursor cells (hSKPs) could be induced to differentiate into osteoblasts [16]. This report demonstrated that the hSKPs

under appropriated induction could be differentiate into corneal epithelial-like cells by using a multiple set of growth factors. The epidermal growth factor (EGF), keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) are believed to play an important role in corneal epithelial cells maintenance and wound healing. Levels of HGF, KGF and EGF mRNAs were increased in rabbit lacrimal gland tissue in response to corneal epithelium wound healing [17]. They are stimulated proliferation, migration of the corneal epithelium [18]. The *in vitro* transdifferentiation process of hSKPs was monitored by the expression of putative stem cell markers (ATP-binding cassette subfamily G member 2; ABCG2, integrin alpha 9; ITGA9 and keratin 19; K19) and specific corneal differentiation markers (keratin 3; K3 and keratin12; K12). It is strongly believed that the potential application of hSKPs may serve as an alternative source of stem cells for ocular surface reconstruction.

MATERIALS AND METHODS

Materials

Cultivation of hSKPs Our medical staff group obtained human skin biopsies with informed consent from healthy donors with ages from 40 to 60 years old. The hSKPs were cultured using the protocol of previously a reported system[16]. Briefly, skin biopsies were dissected into small pieces and placed into 35 mm Petri dish (Corning Costa), cultured in Dulbecco's modified Eagle's medium (DMEM), low glucose medium (Gibco BRL Gaithersburg,MD, USA) and supplemented with 10% fetal bovine serum (Gibco BRL) and 1% penicillin/streptomycin (Gibco BRL). The cultured cells were submerged in medium and incubated in a humidified atmosphere 95% air and 5% CO_2 at 37℃. The medium was changed twice a week until cells became confluent. The confluent cells were harvested by using 0.05% trypsin-ethylenediaminetetraacetic acid (EDTA) (Gibco, BRL).

Methods

Flow cytometry analysis The cellular surface markers of hSKPs were determined by immunofluorescence using a panel of anti-human monoclonal antibodies CD14, CD29, CD34, CD44, CD45, CD68, CD90 and CD105, which were conjugated with fluorescent dye (all reagents from Chemicon international, Temecula, CA, USA). The cultivated hSKPs were harvested from a culture vessel, washed with phosphate buffer saline (PBS; Gibco BRL) and centrifuged at 600 g for 5 minutes at room temperature. The pellet cells were incubated with the panel of anti-human monoclonal antibodies for 30 minutes at 4 ℃ in the dark room, washed three times and resuspended by PBS. Cell suspensions were analyzed using a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA USA).

***In vitro* transdifferentiation of hSKPs into corneal epithelial-like cell** An aliquot of 1×10^5 cells/mL of hSKPs passage 4 was seeded onto a 35mm of Petri dish. The culture cells were submerged in the medium. The control medium included DMEM:Ham's F12 medium (Gibco BRL) which was supplemented with 10% FBS, 5μg/mL of insulin (Sigma-Aldrich, St Louis, MO, USA), 0.5μg/mL of hydrocortisone (Sigma-Aldrich) and 1% penicillin/ streptomycin. The induction medium included all components of the control medium, with the addition of 10 ng/mL of EGF (Sigma-Aldrich), 20 ng/mL of KGF (Sigma-Aldrich) and 20 ng/mL of HGF (Chemicon international). The cultured cells were maintained for 21 days, changing the medium twice a week. Cells were harvested for morphological observation every 7 days.

Immunofluorescence assay The cultured cells were washed with PBS and fixed in cool methanol for 15 minutes. The methanol-fixed cells were washed in PBS, blocked with 3% BSA in PBS for 60 minutes. After washing, they were incubated with 1∶100 anti-K3/12 (AE5 clone; Chemicon international) for 60 minutes, washed with PBS and incubated with 1∶200 labeled-FITC anti-mouse IgG (Chemicon international) for 30 minutes at room temperature. The cells were washed in PBS, counter- stained with 0.002μg/mL of propidium iodide (PI; Sigma-Aldrich) for 15 minutes, washed with PBS and analyzed with an epifluorescence microscope (Te-2000u Eclipse; Nikon, Japan).

Real-time reverse transcription-polymerase chain reaction Total RNA was extracted from cultured cells by using a protocol for TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNase I treatment (Invitrogen) was performed to eliminate genomic DNA from samples. The cDNA was reverse-transcribed by Superscript III (Invitrogen). The PCR amplifications were analyzed by quantitative Real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) in TaqMan® Gene Expression Assay (Applied Biosystems) of K3 (Hs0036508_m1), K12 (Hs00165015_m1), K19 (Hs00761767_s1), ITGA9 (Hs00174408_m1), ABCG2 (Hs00184979_m1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs02758991_g1) as the internal control. All PCR reactions were performed in a real-time PCR 7300 system (Applied Biosystems). The assays were performed as the second step in a two-step RT-PCR. Assays were done in 25 microliter singleplex reactions containing TagMan® Universal PCR Master Mix, 20X TagMan® Gene Expression Assays. The reaction conditions consisted of 45℃ for 30 minutes, 95℃ for 10 minutes, then cycling for 40 cycles of 95℃ for 10 second and 60℃ for 1 minute.

Western blot analysis The cultured cells were washed with cold PBS. The protein lysate was prepared using a previously reported system [19] and transferred into microcentrifuge tubes on ice. The lysed cells were spun at 10 000g for 10 minutes, and the supernatant was immediately

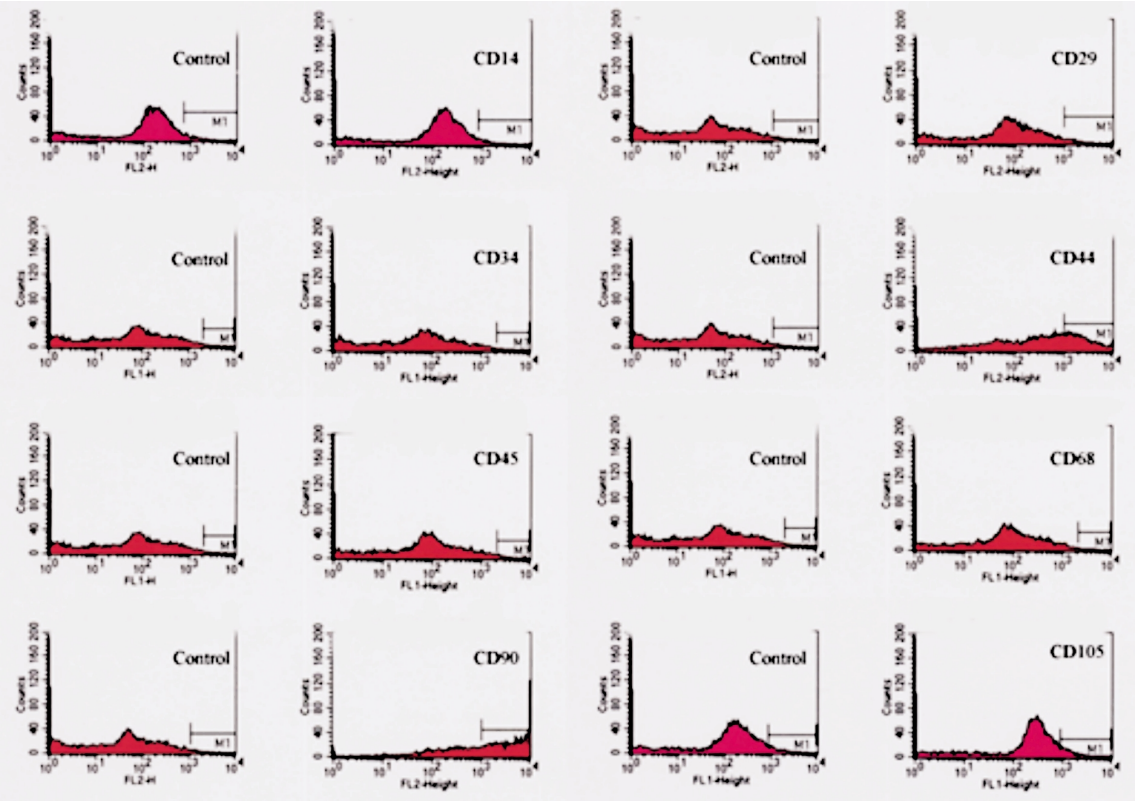

Figure 1 The cellular surface antigens of hSKPs were determined by FACS assay.

collected and stored at -70℃. Protein concentrations in the cell lysates were determined by the Bradford protein assay (BioRad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The 20μg protein solutions were separated by 10% polyacrylamide gel electrophoresis (BioRad Laboratories), and transferred to polyvinyldifluoride (PVDF) membrane. The transfer was monitored by a prestained protein molecular weight marker (BioRad Laboratories). The immunologic detections were determined with anti-K3/12 by LumiGLO Western Blotting Kit (KPL Gaithersburg, Maryland, USA) according to the manufacturer's instructions. Blots were scanned with a flat-screen scanner (Hewlett-Packard, PaloAlto, CA, USA). Banding analysis was performed with Gel Pro Analyzer version 3.1 (Media Cybernetics, USA). The K3 and K12 in the assay were conclusively differentiated by molecular size. The human corneal epithelial cells (HCEC; Cascade Biologics, USA) were used as K3 protein concentration control.

Statistical Analysis All data were presented as the mean± standard deviation (SD). The evaluation significant between different duration of treatment was performed with the Sign rank test for nonparametric analysis. $P<0.05$ was considered statistical significant.

RESULTS

hSKPs Characterization The morphology of hSKPs during expansion were observed under phase contrast microscopy showed a spindle shape, similar to that found in fibroblast and bone marrow-derived stem cells (BMSC). The cellular surface antigens of hSKPs were determined by FACS assay showing positive reaction for CD29, CD44, CD90 and CD105 and negative reaction for CD14, CD34, CD45 and CD68 (Figure 1).

***In vitro* Transdifferentiation of hSKPs into Corneal Epithelial-like Cells** The hSKPs on day 21 of cell culture in induction medium transformed their spindle shape into polyhedral shape with increasing cytoplasm and formed the cluster of polyhedral shape surrounded with spindle shape cells. On day 7 and 14 the culture cells were still appeared as spindle shape like cells in control medium. The putative stem cell genes (K19, ABCG2 and ITGA9) of these cells obviously demonstrated for their expression by using real-time RT-PCR technique. They were significantly declined over the 21 days of cell culture ($P<0.05$; $n=3$) (Table 1, Figure 2). During the transdifferentiation period, the corneal epithelial-like cells were investigated by immunofluorescence, real-time RT-PCR and Western blot assay. The expression of K3 gene was initially increased on day 21 of transdifferentiation. It was in accordance with the result of immunofluorescence with anti-K3/K12 which showed positive result on day 21 (Figure 3). The protein expression of K3 on day 21 was confirmed by Western blot assay. The expression of K3 protein was less than in human corneal epithelial cell, the expression ratios as compared to human corneal epithelial cells were shown to be 0.65± 0.22 (mean ± SD; $n=6$) (Figure 4) The K12 gene and K12 protein were not expressed over 21 days of transdifferentiation.

Table 1 Comparison of genes expressions change between in cultured hSKPs with induced medium and those with control medium at day 21

Genes	The normalized gene expression-to-GAPDH ratio(RQ) of cultured hSKPs at day 21	
	Induced medium(mean±SD)	Control medium(mean±SD)
ABCG2	0.35±0.05	0.73±0.04
ITGA9	0.36±0.05	0.55±0.11
K19	0.35±0.04	0.74±0.01
K3	0.29±0.02	0.00
K12	0.00	0.00

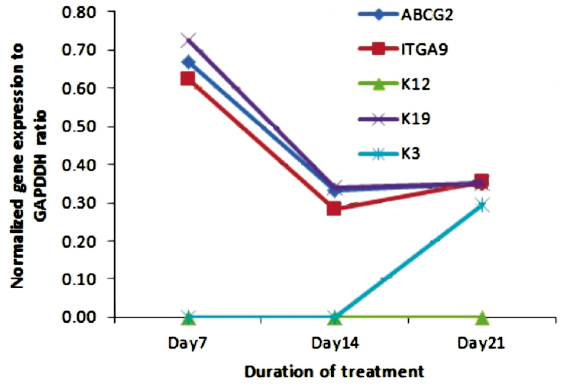

Figure 2 Real-time RT-PCR analysis of the expression of ABCG2, ITGA9, K12, K19 and K3 genes over the 21 days transdifferentiation studies of 3 samples. Treatment duration in 7 days intervals and control are shown as the normalized gene expression-to-GAPDH ratio (RQ).

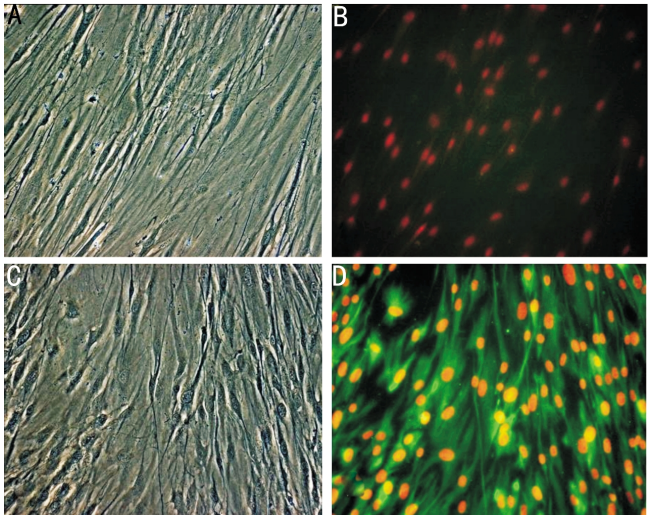

Figure 3 Micrographs of hSKPs culture on day 21 Phase contrast micrographs show spindle -shaped cells in control hSKPs (A) as compared to the polyhedral shape in transdifferentiated hSKPs (C). Immunufluorescence staining of anti-K3/K12 (AE5 clone) of control group (B) and transdifferentiated hSKPs (D) are shown. The red nuclei were counter-stained with propidium iodide (PI). Magnification: ×100.

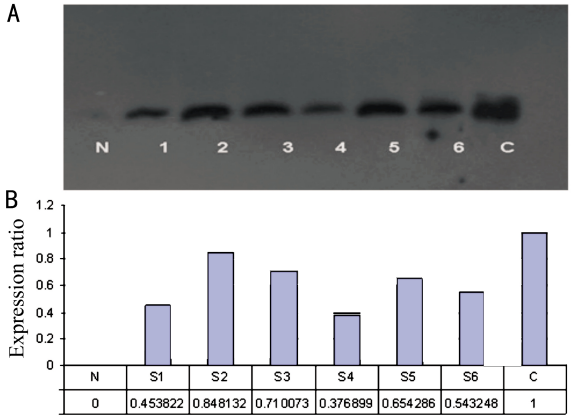

Figure 4 Western blot assay of K3 protein expression (A) and expression ratio of K3 (B) of control group (N), the transdifferentiation studies on day 21 of 6 samples (S1, S2, S3, S4, S5 and S6) as compared to human corneal epithelial cells (C).

DISCUSSION

The examinations on *ex vivo* reconstruction of a corneal epithelium have been performed by using LSC from limbal biopsies. The disadvantage of this technique was the histocompatibility of donor LSC, the risk of tissue injuries in harvested biopsies and not useful for treating bilaterally affected ocular surface disorders. Alternative stem cell sources such as oral mucosal epithelium cells mesenchymal stem cell and immature dental pulp stem cells have been explored for application in corneal epithelium replacement. The authors proposed another stem cell source: the hSKPs harvested from dermal skin, which first isolated from adult skin in 2001 [20]. This report we found, they have spindle shaped-cells like the ones found in fibroblast and mesenchymal stem cells. The hSKPs in this model showed less stemness genes ie, positive in CD44, CD90 and CD105 as like those found in mesenchymal stem cells [21]. In contrast, the porcine skin stem cells showed Oct3/4, Sox2, Stat3 and nanog which pluripotency-related genes [22]. Comparing between hSKPs with mesenchymal stem cell, we found the expression of nestin in hSKPs as previously described by Zhao *et al* [23]. Additionally, hSKPs expressed CD29 (integrin beta 1), which was suggested to be a marker for epidermal stem cells [24] and lacking the expression of hematopoietic progenitor cell markers; CD14, CD34, CD45 and CD68. The multipotentcy of hSKPs has been previously described including neuronal derivatives[20,25-27] and mesoderm derivatives (osteocytes, chondrocytes, smooth muscle cells and adipocytes) [16,20,28,29]. For the study of markers, ABCG2, ITGA9 and K19 are proposed as limbal stem cell markers.

The expression of ABCG2 and K19 in basal layers of a limbus is greater than those found in the basal layer of a central cornea [30]. The ITGA9 is a transmembrane heterodimeric protein, which serves as receptors for cell to cell and cell to an extracellular matrix. It appears in the basal layer of a limbus but disappears in the basal layer of a central cornea and usually appears in retained labeling cells with 5-bromo-2-deoxyuridine [31]. The K19 marker is an intermediate filament, which is suggested to be a marker for the proliferating keratinocyte cells from skin [32,33]. The expression of K19 in limbal basal cells was higher than those found in corneal basal cells[34]. The K3 and K12 markers are widely used to define corneal epithelial cell maturation. Both of these two markers could express in the basal and suprabasal layers of a cornea but not in the basal layer of a limbus[35].

The EGF, KGF and HGF are the major growth factors in this model. The EGF is paracrine growth factors, which stimulated proliferation and migration of corneal epithelial cells by ERK and PI3K/AKT pathway[36]. The KGF and HGF are paracrine growth factors from keratinocyte, which highly expressed in corneal epithelial injury. They are mainly growth factors to regulate corneal epithelial cells and activate Ras-MAPK pathway[37]. The cultivation of hSKPs in the culture plate for 21 days, changes in hSKPs were observed, including the transdifferentiation of hSKPs to corneal epithelial-like cells. The findings were confirmed as shown by the observation of changing the spindle cell shape to polyhedral cell shape. In addition, the loss of stem cell pluripotency in these transdifferentiated cells were demonstrated as shown by decreasing expression of putative stem cell genes (ABCG2, ITGA9 and K19); with the expression of corneal epithelial cell marker (K3) as shown by real-time RT-PCR, immunofluorescence and Western blot. This experiment was designed to last 21 days. Although IGTA9 was slightly elevated on day 21, but it was not statistically significant when compared with the result on day 14. Moreover, the amount of ITGA9 on day 21 was clearly lower than the control on that day.

It is noted that there were no K12 expression in this experiment. Human corneal epithelium, should express the positive label in both K3 and K12. However, our finding in hSKPs cells was in concurrence to that of Nakamura's study *in vivo* on expanded oral mucosal cells in autologous corneal transplantation. The epithelial tissue equivalent, which was generated after two to three weeks of culture, showed cornea-like properties with expression of K3 (but not K12) [8,10]. No K12 expression and the low level of K3 expression in this model might indicate that the transdifferentiated cells were less mature than native corneal epithelial cells. This phenomenal could be due to (1) the studied cells were adult stem cells not embryonic stem cells and (2) the studied cell were collected from other sources rather than corneal stem cells. Therefore, the transdifferentiation of adult stem cells from different source of origin, may express different markers when compared with makers expression in native cell form limbal zone. The negative expression of K12 of these cells in this study could not clearly indicate that hSKPs may or may not transdifferentiate to corneal epithelial cell. However, the K3 positive expression could at least provide a clearer picture of the possibility of hSKPs, to potentially transdifferentiate into corneal epithelial-like cells. Nevertheless, appropriated condition of cell transdifferentiation from different source of tissue into corneal epithelial-like cell as shown in this report, has triggered the future solution of availability of corneal epithelial cell. In conclusion, we demonstrated the presence of cell markers and morphological appearance of corneal epithelial cells as induced in the cells from other tissue (skin). Loss of pluripotency and expressing the corneal epithelial cell marker (K3) occurrence of hSKPs indicated transdifferentiation phenomena of these cells. Hopefully, this study leads to the utility of a valuable stem cell source for corneal disorder treatment.

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