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Characterization of oral keratinocyte stem cells and prospects of its differentiation to oral epithelial equivalents

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Abstract

Objective: Although oral keratinocyte stem cells play a key role in tissue homeostasis, wound healing, and neoplasia, they remain difficult to identify and characterize. The specific aim of the present study is to characterize an oral keratinocyte stem-cell population separated using a magnetic technique. *Material and Methods*: Oral human keratinocytes obtained from keratinized oral mucosa were magnetically separated using a proliferation-related marker, CD71 and $\alpha_6\beta_4$ integrin. The expression of different stem cell markers: CD44H, Nestin, Nanog, Oct 3/4, CD117 was checked by immunofluorescence. The ability of $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ fraction to form oral epithelial equivalents was also assayed. *Results*: Three different oral keratinocyte subpopulations were obtained following magnetic separation: $\alpha_6\beta_4^{pos}$ $CD71^{neg}$, $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ and $\alpha_6\beta_4^{neg}$. Our $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ stem cell fraction was positive for Oct 3/4, CD44H and cytokeratin 19 while Nanog, Nestin and CD117 expression was absent. At the same time, the other two cell fractions $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ and $\alpha_6\beta_4^{neg}$ were negative for all stem cell markers. Also, $\alpha6\beta4^{pos}$ $CD71^{neg}$ fraction was able to regenerate a well stratified and organized oral epithelial equivalent. The distribution of cytokeratin 19 and involucrin in the oral epithelial equivalent reflected the *in vivo* situation in oral gingival epithelium. *Conclusions*: The human gingival $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ fraction was strongly positive for a panel of stem cell markers and could form oral epithelial equivalent. It is also suggested that a magnetic system may be an important tool in acquiring oral keratinocyte stem cells for research.

***Keywords*:** oral keratinocyte, stem cell, magnetic separation, oral mucosa.

Introduction

The oral epithelium is a stratified squamous tissue that renews itself rapidly due to the cells residing in the basal layers. Basal epidermal layers contain a small population of highly proliferative, quiescent cells i.e. stem cells [1]. These cells constantly give rise to transit amplifying (TA) keratinocytes, which undergo terminal differentiation as they migrate to the surface [2]. Oral keratinocyte stem cells (OKSC) play key roles in cell renewal, wound healing and tissue homeostasis but they remain difficult to isolate in a viable state due to the lack of specific surface markers.

Previously [3] we successfully managed to isolate an OKSC population using magnetic separation and two surface markers, α6β4 integrin and CD71. A6β4 integrin is expressed exclusively on the surface of basal keratinocytes, playing a crucial role in hemidesmosomes assembly by binding to laminin-5 in the basement membrane [4]. Actively-cycling epidermal cells such as TA express high levels of CD71 proliferation-related surface marker, while quiescent cells such as stem cells show extremely low levels of CD71 [5]. Based on the two surface markers we separated three populations of basal keratinocytes, $\alpha_6\beta_4^{pos}$ $CD71^{neg}$, $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ and $\alpha_6\beta_4^{neg}$. We also provided convincing evidence that $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ fraction has important stem cell attributes. We showed that this cell fraction represents a quiescent minor population of basal keratinocyte cells, having the highest colony forming efficiency, and the smallest cell size among basal keratinocyte cells. $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ were also positive for keratinocyte stem cell markers p63 and keratin 19 and negative for keratinocyte differentiation markers involucrin and keratin 10.

The purpose of the present study is to further characterize $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ fraction using known stem cells markers and to asses the ability of regenerating its tissue or origin by forming oral epithelia equivalents (OEE).

Material and Methods

Isolation of primary keratinocytes and fibroblasts

All protocols for this study were reviewed and approved by the Research Ethics Committee of Nippon Dental University. Gingival tissues were supplied by patients undergoing oral surgeries at Nippon Dental University Hospital. Informed consent was obtained from the subjects who agreed to participate voluntarily in this study. Keratinized oral mucosa was obtained from patients undergoing tooth extraction. Tissues were cut into small pieces and subjected to enzymatic dissociation in 3 mg/mL Collagenase (Sigma, St. Louis, MO) and 4 mg/mL Dispase (Sigma, St. Louis, MO) at 4^0C for 24 hours. The following day, the epidermal sheet was removed from the connective tissue. To obtain viable single keratinocyte cells, the epidermis was treated with 0.05% trypsin at 37^0C for 30 minutes. The medium used for keratinocyte cells growth was EpiLife® medium (Cascade Biologics, Portland, OR) supplemented with 1.2 mM calcium, EpiLife® Defined Growth Supplements (EDGS) (Cascade Biologics, Portland, OR), 0.250 μg/mL Fungizone and 0.250 mg/mL Kanamycin. The cells were cultured in 35 mm diameter dishes precoated with human collagen type IV (20 μg/mL) (Sigma, St. Louis, MO) at 36^0C in an atmosphere of 5% carbon dioxide (CO_2). Dermal sheets were treated with 0.05% trypsin for 30 minutes at 37^0C and single viable fibroblasts were resuspended in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) at 37^0C in an atmosphere of 5% carbon dioxide.

Magnetic cell sorting

Magnetic separation (Figure 1) was performed following the manufacturer's instructions. Briefly, the keratinocytes were incubated together with mouse monoclonal integrin-$\alpha_6\beta_4$ antibody. The cells were further reacted with goat anti-mouse IgG MicroBeads and the cell suspension was loaded into a column placed in the magnetic field of a MACS® Separator (Miltenyi Biotec Inc., CA, USA). The magnetically-labeled cells were retained in the column as the $\alpha_6\beta_4^{pos}$-fraction while the unlabeled cells ran through the column representing the $\alpha_6\beta_4^{neg}$-fraction. Further, the $\alpha_6\beta_4^{pos}$-fraction was magnetically labeled with CD71 MicroBeads and subjected to the same procedure of magnetic cell sorting. The magnetically-labeled $CD71^{pos}$ cells were retained in the column, while the unlabeled $CD71^{neg}$ cells ran through the column. The $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ fraction was assumed to be the putative stem-cell population.

Antibodies

Mouse monoclonal [450–30A] antibody to Integrin alpha 6 beta 4 (Abcam, Tokyo, Japan) conjugated with Fluorescein isothiocyanate (FITC); mouse monoclonal anti-involucrin antibody (Sigma-Aldrich, Germany); mouse monoclonal anti-cytokeratin 19 antibody (Abcam, Tokyo, Japan); anti-Oct 3/4, anti-CD44H (R&D Systems Inc., Minneapolis, USA); anti-Nanog (Sigma-Aldrich, St Louis, MO); anti-CD117 (Miltenyi Biotec, Bergich Gladbach, Germany); anti-Nestin (Abcam, Cambridge, MA, USA) were used at 1:200 for immunocytochemistry or immunohistochemistry staining. As a secondary antibody, Alexa Fluor 568-conjugated donkey anti-mouse IgG (Invitrogen, Eugene, OR) was used to detect mouse primary antibodies. DAPI-nucleic acid stain (Invitrogen, Eugene, OR) was used to stain nuclei. MicroBeads conjugated to monoclonal anti-human CD71 antibodies (isotype mouse IgG2a) (Miltenyi Biotec Inc., CA, USA) were used at a concentration of 20 μL/10^5 cells for magnetic separation. Goat Anti-Mouse IgG MicroBeads (Miltenyi Biotec Inc., CA, USA) were used according to the manufacturer's instructions as a secondary antibody for magnetic separation.

Immunocytochemistry

Different cell fractions were cultured in four-chamber slides (5000 cells/slide) (Nalge Nunc International Naperville, IL, USA). The cells were routinely fixed with 4% paraformaldehyde and then labeled with the different primary antibodies, followed by Alexa Fluor® 568-conjugated secondary antibody. After each antibody layer, the samples were washed with PBS, and the stained cells were observed under a confocal scanning laser fluorescence microscope.

Oral epithelia equivalents

Oral epithelia equivalents (OEE) were obtained using a previously established Transwell system (Transwell® Permeable supports, Costar Life Sciences, NY, USA) consisting of a purified collagen solution (Cellmatrix, Nitta Gelatin Inc., Osaka, Japan) populated with 5×10^4 human gingival fibroblasts per well-known as dermal equivalent. Dermal equivalents were cultured in DMEM with 10% FBS for seven days to allow gel contraction. Subsequently 5×10^4 oral keratinocyte stem cells were seeded per collagen gel. The cells were grown submerged into EpiLife® medium supplemented with EDGS for four days and then raised to an air-liquid interface for another 10 days. Immunohistochemistry staining was performed as described previously. Both keratinocytes and fibroblasts used for reconstituting oral epithelia equivalents were obtained from the same patient.

Immunohistochemistry staining

The OEE was fixed in 10% paraformaldehyde for 24 hours, at 4^0C. Following dehydration in ethanol, the samples were embedded in paraffin and frontally sectioned at a thickness of 3 μm. Serial sections were also immunohistochemically processed and stained for cytokeratin 19 and involucrin.

Results

Separation of basal keratinocytes based on $\alpha_6\beta_4$ integrin and CD71 expressions

As described in detailed previously [3], we managed to isolate three subpopulations of keratinocytes using magnetic separation: $\alpha_6\beta_4^{pos}$ $CD71^{neg}$, $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ and $\alpha_6\beta_4^{neg}$ (Figure 1.). We showed that the $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ fraction is enriched for oral keratinocyte stem cells.

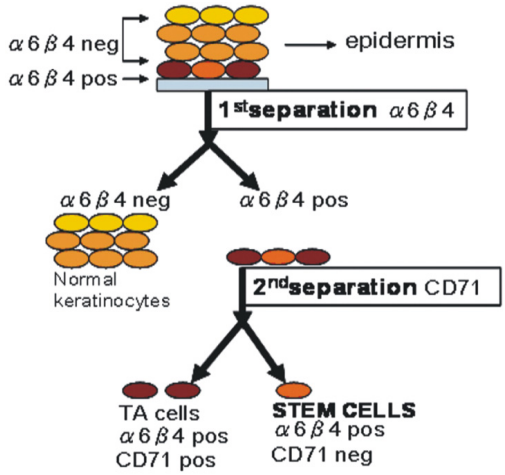

Figure 1 – ***Magnetic separation: α6β4 integrin, expressed exclusively on the surface of basal keratinocytes was used for separating basal keratinocytes from the keratinocytes residing in the upper layers of the epidermis. CD71, a proliferation-related surface marker was used for separating quiescent cells such as stem cells from actively-cycling epidermal cells such as transit amplifying cells (TA).***

Expression of different stem cell markers

Following magnetic separation we examined by immunostaining the expression of CD44, cytokeratin 19; Oct 3/4; Nestin; Nanog and CD117 (Figure 2) in all three keratinocyte subpopulations. Our putative stem cell fraction $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ was strongly positive for CD44, cytokeratin 19 and Oct 3/4 while Nanog, Nestin and CD117 expression was absent (Figure 2). The other two cell fractions: $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ and $\alpha_6\beta_4^{neg}$ stained negative for all six stem cell markers (data not shown).

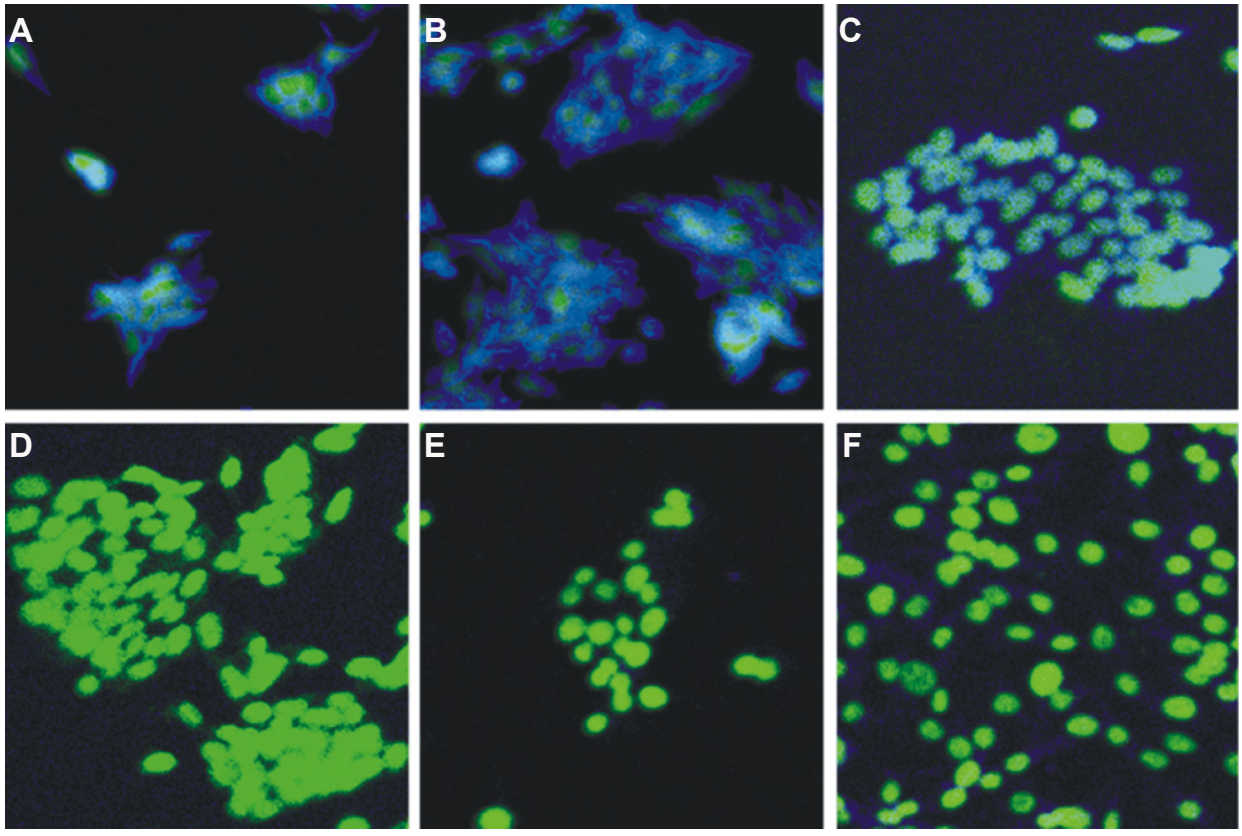

Figure 2 – ***Immunofluorescence of magnetically sorted α6β4^{pos} CD71^{neg} – keratinocyte stem cell fraction: the cells show strong positive staining for (A) CD44 (blue); (B) Cytokeratin 19 (blue); the arrow shows perinuclear distribution of (C) Oct 3/4 (blue). The cells are negative for (D) Nestin; (E) Nanog; (F) CD117. The nuclei of the cells are stained with DAPI (shown in green).***

Immunohistochemical staining for oral epithelia equivalents

After 14 days in culture, OEE (Figure 3A) were stained with antibodies to markers of differentiation and stem cells. Involucrin, a marker of terminal differentiation, was strongly expressed in the upper layers of the OEE and absent in the lower ones (Figure 3B). At the same time immunostaining with cytokeratin 19, showed that the keratin is present only in the lower layers while being completely absent in the upper layers (Figure 3C). This data shows that OEE has a highly organized multilayered structure resembling normal oral epithelia with keratinocyte cells from the upper layers being in a state of terminal differentiation and the ones from the basal layers having stem cells attributes.

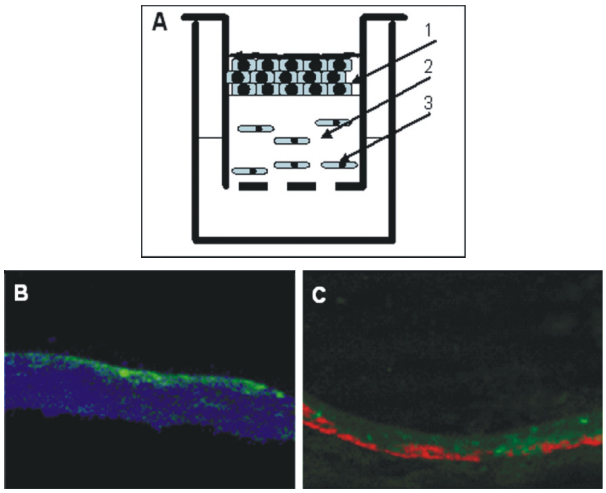

Figure 3 – ***Oral epithelia equivalent: (A) schematic representation; keratinocytes (1) are growing in ordered multilayers on collagen gel (2) with human fibroblasts (3) being used as feeder layer in the lower part of the culture insert. (B) Immunostaining of involucrin (green), the data clearly shows that only the upper layers of OEE express the differentiation marker involucrin (C) Immunostaining of cytokeratin 19 (red); note that CK19 – stem cell marker expressing cells are present in the basal layers of the OEE where the undifferentiated cells reside.***

Discussion

Recently techniques for magnetic cell isolation have been dramatically improved and have become a popular tool for the separation of target cells [6, 7]. Magnetic particle technology has provided an efficient, simple, and rapid cell-separation method [8]. Examples of magnetic cell separation include sperm selection based on sperm apoptotic markers [7], isolation of human B-lymphocytes [9] or separation of rare progenitor cells from human umbilical cord blood [10, 11]. Previously in our laboratory we managed to isolate CD-117 positive dental pulp mesenchymal stem cells using magnetic microbeads (data not shown). In this study, we used a magnetic cell sorter to isolate target cells from cell suspensions using immunomagnetic particles. Since magnetic separation requires fewer cells than a flow cytometer, this procedure is suitable for a small number of target cells, such as OKSCs from a small gingival biopsy. Most importantly, the technique can be easily applied in clinics and used for oral tissue engineering.

After separation using the two markers the expression of different stem cell markers was examined in all three magnetically separated populations. Previous work has shown a multitude of markers that can be found in stem cells but up to this date there are no specific markers for epidermal keratinocyte stem cells in general and for OKSCs in particular [12]. Therefore, in the present study we chose a panel of widely accepted stem cell markers: CD44H, Nestin, Nanog, Oct 3/4, CD117 and we checked for their availability in our magnetic separated the $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ stem cell fraction. CD44H is a type 1 transmembrane glycoprotein used to identify specific types of mesenchymal stem cells [13, 14]. Nestin is a Class VI intermediate filament expressed in the developing central nervous system (CNS) in early embryonic neuroepithelial stem cells [15]. Nanog is a homeodomain-bearing transcriptional factor specific to early embryos and pluripotential stem cells [16]. Oct 3/4 is renowned as a transcription factor expressed by undifferentiated embryonic stem cells and embryonic germ cells [17]. CD117 serves as the receptor for stem cell factor (SCF) and is expressed in hematopoietic stem and progenitor cells [18]. Cytokeratin 19 (CK19) identifies specific pancreatic epithelial cells that are progenitors for islet cells and ductal cells. Our data shows that $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ stem cell fraction is positive for CD44H, cytokeratin 19 and Oct 3/4 while Nanog, Nestin and CD117 expression is absent. At the same time the other two magnetic separated fractions $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ and $\alpha_6\beta_4^{neg}$ were negative for all stem cell markers.

Another strong line of evidence that $\alpha6\beta4^{pos}$ $CD71^{neg}$ fraction contains oral keratinocyte stem cells is its ability to regenerate a well-stratified and organized oral epithetlial equivalent. Our data is consistent with previous studies involving skin keratinocyte stem cells. The basal layer of OEE evidenced stronger immunoreactivity for cytokeratin 19 (a marker for cells in an undifferentiated state) than was observed in other epithelial layers. At the same time, OEE immunostaing for involucrin (a marker of keratinocytes committed to the differentiation process) was confined only to the superficial layer while the other epithelial layers did not show immunoreactivity. The distribution of cytokeratin 19 and involucrin reflects the *in vivo* situation in oral gingival epithelium. However, the data showed that the obtained OEE is still immature. Further study is in progress to develop the matured OEE.

In the present study, we have shown that oral basal keratinocytes with the phenotype $\alpha6\beta4^{pos}$ $CD71^{neg}$ are positive for certain stem cells markers and can regenerate their tissue of origin. The ability to isolate and characterize an oral keratinocyte stem cell population has important implications in defining the role of these cells in epidermal differentiation, and in studies of epithelial disorders including carcinogenesis.

Conclusions

We have shown that well characterized OKSC population can be successfully isolated using a magnetic cell-sorting system. Transfer of this new approach to clinics may lead to significant improvement cell and tissue engineering and in developing better-organized oral human epithelia.

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