Journal of
PERIODONTAL RESEARCH

J Periodont Res 2010; 45: 703–708

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2010.01284.x

Magnetic separation and characterization of keratinocyte stem cells from human gingiva

B. Calenic[1], N. Ishkitiev[1], K. Yaegaki[1], T. Imai[1], Y. Kumazawa[2], M. Nasu[3], T. Hirata[1]

[1]Department of Oral Health, [2]Oral Maxillofacial Surgery and [3]Research Center for Odontology, Nippon Dental University, Tokyo, Japan

Calenic B, Ishkitiev N, Yaegaki K, Imai T, Kumazawa Y, Nasu M, Hirata T. Magnetic separation and characterization of keratinocyte stem cells from human gingiva. J Periodont Res 2010; 45: 703–708. © 2010 John Wiley & Sons A/S

Background and Objective: Although keratinocyte stem cells play a key role in tissue homeostasis, wound healing and neoplasia, they remain difficult to identify and characterize. The purpose of this study was to isolate and characterize an oral keratinocyte stem-cell population.

Material and Methods: Oral human keratinocytes obtained from keratinized oral mucosa were magnetically separated using $\alpha_6\beta_4$ integrin and a proliferation-related marker, CD71. The isolated cell fractions were analyzed for cell size, cell cycle stage (using flow cytometry) and colony-forming ability. The expression of stem cell markers p63 and cytokeratin 19 and of differentiation markers cytokeratin 10 and involucrin was checked using immunocytochemical analysis.

Results: The stem cell $\alpha_6\beta_4^{pos}$ CD71^{neg} fraction had the smallest cell size compared with $\alpha_6\beta_4^{pos}$ CD71^{pos} and $\alpha_6\beta_4^{neg}$ fractions [780.7 ± 141.5 (pixels), 1422.9 ± 264.6 (pixels) and 3844.4 ± 220.1 (pixels) respectively, $p < 0.01$; analysis of variance (ANOVA)]. Also, the $\alpha_6\beta_4^{pos}$CD71^{neg} subpopulation consistently had the highest colony-forming ability among the three cell fractions (126.2 ± 21.7 vs. 32.8 ± 4.5 vs. 12.4 ± 2.1 compared with $\alpha_6\beta_4^{pos}$ CD71^{pos} and $\alpha_6\beta_4^{neg}$ subpopulations, respectively, $p < 0.01$; ANOVA). Moreover, the $\alpha_6\beta_4^{pos}$ CD71^{neg} fraction contained more quiescent cells and fewer actively cycling cells than the $\alpha_6\beta_4^{pos}$ CD71^{pos} cell fraction. The candidate stem cells were positive for cytokeratin 19 and p63 keratinocyte stem cell markers, while differentiation markers such as cytokeratin 10 or involucrin were absent.

Conclusion: The human gingival $\alpha_6\beta_4^{pos}$ CD71^{neg} cell fraction, separated by a magnetic system, demonstrated several characteristics of gingival keratinocyte stem cells. It is also suggested that a magnetic system may be an important tool in acquiring oral keratinocyte stem cells for research.

Ken Yaegaki, DDS, PhD, Department of Oral Health, Nippon Dental University, 1-9-20 Fujimi, Chiyoda-ku, Tokyo, 102-8159 Japan
Tel: +81 3 3261 8791
Fax: +81 3 3261 8796
e-mail: yaegaki-k@tky.ndu.ac.jp

Key words: oral keratinocyte; stem cell; magnetic separation; oral mucosa

Accepted for publication January 27, 2010

Gingival epithelium is a stratified, rapidly renewing tissue in which the epithelial cells in the upper layers are continuously lost and replaced with newly proliferated cells originating in the basal layer. Under normal conditions the basal epidermal layer contains 1–10% keratinocyte stem cells, 50% transit amplifying (TA) cells and 40% postmitotic differentiating cells in early stage keratinization (1). *In vivo*, oral keratinocyte stem cells (OKSCs) form a minor population of basal cells, generally quiescent, with the capacity for self-renewal and a greater proliferative potential over their lifetime (2). These cells constantly give rise to TA cells, which are actively cycling basal cells with a limited lifespan and a lesser proliferative potential. Although OKSCs play crucial roles in cell

renewal, tissue homeostasis, wound healing and neoplasia, they remain poorly characterized and difficult to isolate in a viable state. The availability of suitable OKSC markers and separation techniques would clearly facilitate the isolation and characterization of OKSCs.

Among the most intensively studied molecules of the keratinocyte cell surface is the integrin family of cell-adhesion receptors. Basal cells, including OKSCs, adhere to the basement membrane via adhesion molecules known as integrins. In normal epithelium, $\alpha_6\beta_4$ integrin is expressed exclusively on the surface of basal keratinocytes, playing a crucial role in hemidesmosome assembly by binding to laminin-5 in the basement membrane (3). Thus, $\alpha_6\beta_4$ may provide a suitable marker for OKSCs. However, this marker alone cannot completely distinguish OKSCs from TA, as $\alpha_6\beta_4$ integrin is expressed in both cell types (4). It is well known that keratinocyte stem cells are slow-cycling cells, unlike their direct progeny, TA, which are rapid-cycling cells. Actively cycling epidermal cells, such as TA, express high levels of the proliferation-related surface marker CD71, a transferrin receptor, while quiescent cells, such as OKSCs, show extremely low levels of CD71 (5). Hence, CD71 can be a marker for further separation between OKSCs and TA cells.

In recent years, techniques for magnetic cell separation have been dramatically improved and have become a popular tool for the isolation of target cells because of their more rapid and simpler methodology compared with cell sorting using a flow cytometer (6,7). Moreover, because 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. The objective of our study was to isolate and to characterize an OKSC population from human gingiva for certain stem-cell and differentiation markers. To the best of our knowledge, this is the first study to separate a cell population enriched for OKSCs using a magnetic cell-sorting technique based on $\alpha_6\beta_4$ integrin and CD71 markers.

Material and methods

Isolation and culture of primary keratinocytes

This study was reviewed and approved by the Research Ethics Board of Nippon Dental University. Keratinized oral mucosa was obtained from patients undergoing tooth extraction and was processed within 2 h of collection. In order to avoid any interference of the enzymatic treatment with the expression of different markers in keratinocytes, we chose a well-established protocol for isolating oral primary keratinocytes (4). Tissues were rinsed with phosphate-buffered saline (PBS), cut into smaller pieces and subjected to enzymatic dissociation in 4 mg/mL of Dispase II (Sigma, St Louis, MO, USA) and 3 mg/mL of collagenase (Sigma) for 24 h at 4°C. After treatment, the epidermis was removed from the connective tissue. To obtain viable single keratinocyte cells, the epithelial sheets were treated with 0.05% trypsin for 30 min at 37°C. The cells were resuspended in EpiLife® medium (Cascade Biologics, Portland, OR, USA) supplemented with 1.2 mM calcium, EpiLife® Defined Growth Supplements (Cascade Biologics), 0.250 μg/mL of fungizone and 0.250 mg/mL of kanamycin. The cells were cultured in 35-mm-diameter dishes precoated with human collagen type IV (20 μg/mL) (Sigma) at 36°C in an atmosphere of 5% CO_2.

Antibodies

Mouse monoclonal [450-30A] antibody to integrin $\alpha_6\beta_4$ (Abcam, Tokyo, Japan) conjugated with fluorescein isothiocyanate was used at a concentration of 10 μL per 10^5 cells for flow cytometry analysis and at a dilution of 1:200 for immunocytochemistry staining. Mouse monoclonal anti-involucrin (Sigma-Aldrich, Germany), mouse monoclonal anti-cytokeratin 10 (Acris GmbH, Hertford, Germany), mouse monoclonal anti-p63 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse monoclonal antibody [12G10] to integrin β_1 (Abcam), and mouse monoclonal anti-cytokeratin 19 (Abcam) were used at dilutions of 1:200 for immunocytochemistry staining. As a secondary antibody, Alexa Fluor® 568-conjugated donkey anti-mouse IgG (Invitrogen, Eugene, OR, USA) was used to detect mouse primary antibodies. SYBR Green 1 (Trevigen, Gaithersburg, MD, USA) was used to stain nuclei. Microbeads conjugated to monoclonal anti-human CD71 (isotype mouse IgG2a) (Miltenyi Biotec, Inc., Auburn, CA, USA) were used at a concentration of 20 μL per 10^5 cells for magnetic separation. Goat anti-mouse IgG MicroBeads (Miltenyi Biotec Inc.) were used according to the manufacturer's instructions as a secondary antibody against mouse anti-$\alpha_6\beta_4$ for magnetic separation.

Magnetic cell sorting

The cells were incubated with mouse monoclonal integrin $\alpha_6\beta_4$ [450-30A] fluorescein isothiocyanate-conjugated antibody (Abcam). After removing excess antibody, the cells were further reacted with goat anti-mouse IgG MicroBeads (Miltenyi Biotec Inc.), then the cell suspension was loaded into a column placed in the magnetic field of a MACS® Separator (Miltenyi Biotec Inc.). The unlabeled cells passed through the column and represented the $\alpha_6\beta_4$-negative ($\alpha_6\beta_4^{neg}$) fraction, while the magnetically labeled cells, representing the $\alpha_6\beta_4$-positive ($\alpha_6\beta_4^{pos}$) fraction, were retained in the column. Two to three days after the first separation the $\alpha_6\beta_4^{pos}$ cell fraction was magnetically labeled with CD71 MicroBeads and subjected to the same procedure of magnetic cell sorting. The magnetically labeled CD71-positive ($CD71^{pos}$) cells were retained in the column, while the unlabeled CD71-negative ($CD71^{neg}$) cells passed through the column. After the two magnetic separations the $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ fraction was assumed to be the putative stem-cell population.

Immunocytochemistry

Magnetically separated cells were subcultured in four-chamber slides (5000 cells per slide) (Nalge Nunc International, Naperville, IL, USA).

After fixing with 4% paraformaldehyde the slides were labeled with the different primary antibodies, followed by Alexa Fluor® 568-conjugated secondary antibody. The samples were washed three times with PBS after each antibody layer, and the stained cells were observed under a confocal scanning laser fluorescence microscope.

Flow cytometry

After trypsinization with 0.025% trypsin solution, newly isolated keratinocytes were stained with appropriate primary antibodies for 1 h at room temperature followed by Alexa Fluor® 568-conjugated secondary antibody. After each antibody layer the cells were washed three times with PBS. Unlabeled cells were used as a negative control. For each experiment, 2000 cells were analyzed using Guava EasyCyte flow cytometry (Guava Technologies, Hayward, CA, USA). Data acquisition and analysis were performed using GUAVA CYTOSOFT software.

Cell cycle analysis

After each complete magnetic separation the $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ fraction was incubated in 200 µL of Guava® Cell Cycle reagent for 10 min and then analyzed using Guava EasyCyte flow cytometry. The reagent contains propidium iodide, which labels cellular DNA and can discriminate between different stages of the cell cycle. Data acquisition and analysis were performed using GUAVA CYTOSOFT software.

Colony-forming efficiency analysis

We plated 5000 magnetically separated cells ($\alpha_6\beta_4^{pos}$ $CD71^{neg}$, $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ and $\alpha_6\beta_4^{neg}$ fractions) into six-well plates (Costar, Corning, Eugene, OR, USA) previously coated with human type IV collagen (20 µg/mL) (Sigma). The cells were cultured for 10 d in EpiLife® Defined Growth Supplements and then fixed in 4% paraformaldehyde and stained with 2% crystal violet. Colonies of more than 20 cells were counted separately using Cell Analyst® (AssaySoft, Inc., Fountain Valley, CA, USA). The assay was performed five times.

Cell size analysis

Using a light microscope, photographs of different fractions of cells were taken and the images were analyzed using Cell Analyst® (AssaySoft, Inc.). This assay was performed five times, with 50 cells being counted separately on each occasion.

Results

Separation of basal keratinocytes based on $\alpha_6\beta_4$ integrin expression

Only a small fraction of the newly isolated human oral basal keratinocytes (8.1 ± 0.3%, mean ± standard deviation, five independent experiments) was found to be $\alpha_6\beta_4$ positive by flow cytometric analysis (Fig. 1). This result agrees with the results of previously published studies (3) on the expression of $\alpha_6\beta_4$ integrin and keratinocyte stem cells in human skin *in vivo*.

Expression of stem cell and differentiation markers

Following magnetic separation, we examined, by immunostaining, the expression of keratinocyte stem cell markers p63 and keratin 19, and of keratinocyte differentiation markers involucrin and keratin 10. $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ cells were positive to p63 and keratin 19, $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ cells were positive to keratin 10 and 19, and $\alpha_6\beta_4^{neg}$ cells were strongly positive to keratin 10 and involucrin (Fig. 2).

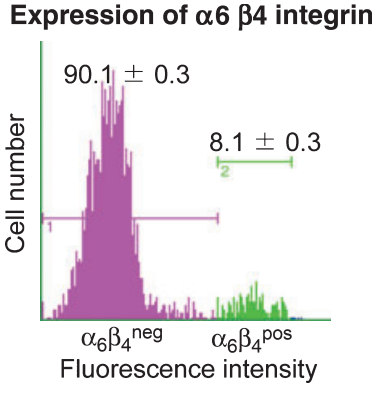

Fig. 1. Flow-cytometric analysis of the integrin $\alpha_6\beta_4$ on newly isolated human oral keratinocytes: $\alpha_6\beta_4$ was detected using Alexa Fluor® 568. Marker 1 (pink) shows that the majority of cells are $\alpha_6\beta_4$ negative, while marker 2 (green) indicates that a minor population of basal keratinocytes is $\alpha_6\beta_4$ positive. Each data point represents the mean ± standard deviation of five independent experiments.

Cell cycle

Immediately after CD71 magnetic separation, the $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ fraction contained more resting cells (G_0/G_1 phase) than the $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ cell fraction (65.9 ± 1.1 vs. 51.8 ± 0.6 respectively, $p < 0.01$; analysis of variance (ANOVA)]. Also, the percentage of actively cycling cells was significantly higher in the $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ cell fraction than in the $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ fraction (for S phase: 24.4 ± 0.5 vs. 16.16 ± 0.2 respectively, $p < 0.01$ ANOVA; for G2/M phase: 20 ± 1.4 vs. 10.18 ± 0.5, respectively, $p < 0.01$ ANOVA) (Fig. 3).

Colony-forming efficiency

The colony-forming ability of the $\alpha_6\beta_4^{pos}$ $CD71^{neg}$, $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ and $\alpha_6\beta_4^{neg}$ fractions was determined after 2 weeks in culture, in five separate experiments. The results showed that $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ cells consistently gave rise to higher colony numbers than the $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ fraction (126.2 ± 21.7 vs. 32.8 ± 4.5 respectively, $p < 0.01$, ANOVA) and than the $\alpha_6\beta_4^{neg}$ fraction (126.2 ± 21.7 vs. 12.4 ± 2.1respectively, $p < 0.01$, ANOVA). These results suggest that the majority of proliferating cells were in the $\alpha_6\beta_4^{pos}$ $D71^{neg}$ cell fraction (Fig. 4A,B). The $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ fraction consistently gave rise to higher colony numbers than the other fractions, indicating that $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ was enriched in colony-forming cells.

Cell size

Following magnetic separation based on $\alpha_6\beta_4$ expression and on CD71 expression, the three cell fractions $\alpha_6\beta_4^{pos}$ $CD71^{neg}$, $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ and

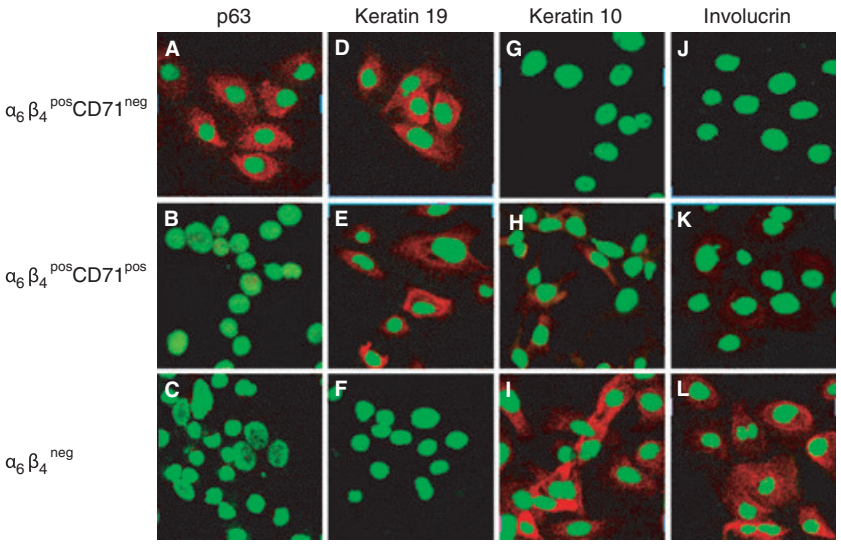

Fig. 2. Immunofluorescence of magnetically sorted oral human keratinocytes. $\alpha_6\beta_4^{pos}$ $CD71^{neg}$, $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ and $\alpha_6\beta_4^{neg}$ cells were stained with antibodies to p63, keratin 19, keratin 10 and involucrin (red), and the nuclei were counterstained with SYBR Green. (A–C) p63 stain for the three cell fractions; (D–F) keratin 19 stain for the three cell fractions; (G–I) keratin 10 staining for the three cell fractions; (J–L) involucrin staining for the three cell fractions. $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ cells were positive to p63 and keratin 19, $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ cells were positive to keratin 10 and 19, and $\alpha_{rm6}\beta_4^{neg}$ cells were strongly positive to keratin 10 and involucrin.

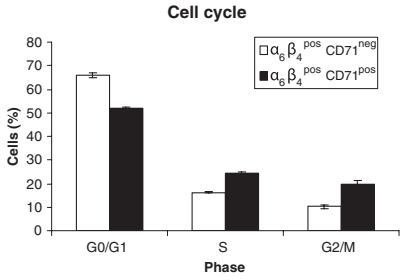

Fig. 3. Cell-cycle analysis of different fractions of oral human keratinocytes after magnetic separation with $\alpha_6\beta_4$ and CD71. Analysis of the two fractioned groups clearly shows that the candidate oral keratinocyte stem cell (OKSC) population ($\alpha_6\beta_4^{pos}$ $CD71^{neg}$) comprises more quiescent cells (G0/G1 phase) and fewer actively cycling cells (S and G2/M phases) than the transit amplifying (TA) candidate cells ($\alpha_6\beta_4^{pos}$ $CD71^{pos}$). Each data point represents the mean ± standard deviation of five independent experiments.

$\alpha_6\beta_4^{neg}$ were analyzed for their size using cell-analysis software. The mean cell sizes for the separated samples were 780.7 ± 141.5 [pixel (px)], 1422.9 ± 264.6 (px) and 3844.4 ± 220.1 (px) for $\alpha_6\beta_4^{pos}$ $CD71^{neg}$, $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ and $\alpha_6\beta_4^{neg}$, respectively ($p < 0.01$, ANOVA) (Fig. 5A). These results are correlated with colony-forming ability, the cells showing an increase in colony numbers with a decrease in the cell size. (Fig. 5B).

Discussion

Regardless of their tissue of origin, all epithelial cells share similar cellular and molecular characteristics (8). In human epithelia the basal epidermal layer is composed of three distinct types of keratinocytes: keratinocyte stem cells, TA cells (i.e. the progeny of stem cells) and postmitotic differentiating cells found in different stages of keratinization. While isolating and characterizing OKSCs would contribute to regenerative dentistry, the lack of appropriate methods or cell-surface markers has made this objective difficult to achieve.

Recent magnetic particle technology has provided a simple, rapid and efficient cell-separation method (9). Successful examples of magnetic cell separation include isolation of rare progenitor cells from human umbilical cord blood (10) (11), isolation of human B lymphocytes (12) and magnetic sperm selection based on sperm apoptotic markers (7). In this study we used a magnetic cell sorter (MACS) to isolate target cells from cell suspensions using immunomagnetic particles (MACS microbeads). An important feature is that the MACS microbeads do not interfere with proliferation assays: both the enriched and depleted fractions of cells can therefore be used in culture immediately following separation (13). Most importantly, the procedure can be easily applied in clinics and used for oral tissue engineering.

Using MACS we managed to isolate physically three populations of basal keratinocytes, $\alpha_6\beta_4^{pos}$ $CD71^{neg}$, $\alpha_6\beta_4^{pos}$ $CD71^{pos}$ and $\alpha_6\beta_4^{neg}$, based on the two surface markers $\alpha_6\beta_4$ integrin and CD71, and we provided convincing evidence that the $\alpha_6\beta_4^{pos}$ $CD71^{neg}$ fraction is enriched for OKSCs. Our results are consistent with previously published studies on keratinocyte stem cells of human skin (14–16), human corneal keratinocytes (17) and rodent skin (18,19). As $\alpha_6\beta_4$ integrin plays an important role in the adhesion between the basement membrane and OKSCs, we chose $\alpha_6\beta_4$ as a marker for stem-cell isolation. Our data show that the $\alpha_6\beta_4^{pos}$ fraction represents a minor subpopulation of approximately 8% of the basal layer, consistent with previous studies showing that 1–10% of skin epithelium is composed of stem cells (20). For further separation between actively cycling TA cells ($CD71^{pos}$) and quiescent OKSCs ($CD71^{neg}$) we used a proliferation-related marker, CD71. After separation using the two markers, the expression of stem-cell markers and keratinocyte markers was examined in all three magnetically separated cell populations. Previous authors have identified a multitude of markers that can be found in keratinocyte stem cells but, to date, there are no specific markers for epidermal keratinocyte stem cells in general and for OKSCs in particular (21). Certain surface markers for stem cells in the neural system, for example, CD133 (22), and in the hematopoietic system, for example, CD34 (23), may

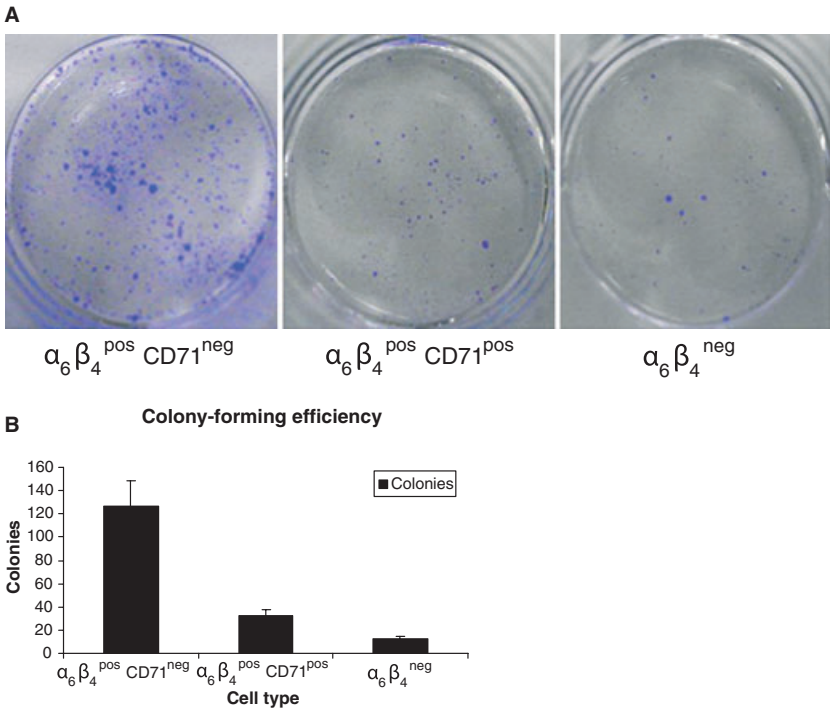

Fig. 4. Colony-forming efficiency. (A) After magnetic separation, 5000 cells from each of the $\alpha_6 beta_4^{pos}$ CD71^{neg}, $\alpha_6\beta_4^{pos}$ CD71^{pos} and $\alpha_6\beta_4^{neg}$ populations were plated for 2 weeks in culture. The colony numbers were determined by staining with crystal violet. Colonies of more than 20 cells were counted separately using Cell Analyst®. (B) The $\alpha_6\beta_4^{pos}$ CD71^{neg} fraction showed significantly higher colony numbers than the $\alpha_6\beta_4^{pos}$ CD71^{pos} and $\alpha_6\beta_4^{neg}$ fractions. Each data point represents the mean ± standard deviation of five independent experiments.

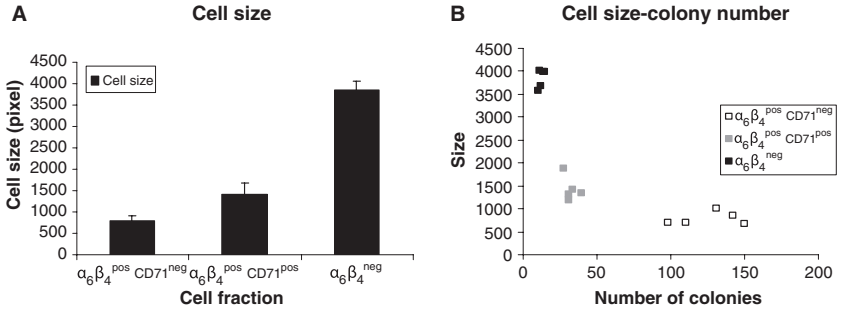

Fig. 5. Cell-size analysis. (A) The $\alpha_6\beta_4^{pos}$ CD71^{neg} cell fraction was found to have the smallest cell size while the $\alpha_6\beta_4^{neg}$ fraction had the largest cell size among the three magnetically separated fractions. Each data point represents the mean ± standard deviation of five independent experiments. (B) Linear regression analysis showing the relationship between colony numbers and the size of the three magnetically separated fractions ($p = 0.001$).

be used as skin stem cell markers, while other studies stress the importance of Notch (24) and c-Myc (25,26) signaling in skin stem cell differentiation. However, it remains to be determined if these markers and pathways are specific to OKSCs. As it is widely accepted that p63 (27,28) and cytokeratin 19 (29) are good candidates for human epidermal stem cell markers, we chose these two markers for characterization of OKSCs. Keratinocytes can be inferred to be in different stages of differentiation based on their expression of cytokeratin 10 and involucrin (30,31). Our data show that p63 is present in the $\alpha_6\beta_4^{pos}$ CD71^{neg} fraction and absent in the other two. Cytokeratin 19 stained both $\alpha_6\beta_4^{pos}$ CD71^{neg} and $\alpha_6\beta_4^{pos}$ CD71^{pos} cells, while the keratinocyte-differentiation markers involucrin and keratin 10 were absent from $\alpha_6\beta_4^{pos}$ CD71^{neg} cells.

The $\alpha_6\beta_4^{pos}$ CD71^{neg}, $\alpha_6\beta_4^{pos}$ CD71^{pos} fractions were also analyzed for their cell-cycle activity. The results showed that the $\alpha_6\beta_4^{pos}$ CD71^{neg} cell fraction was relatively slow-cycling, with significantly more cells in the G0/G1 phase and significantly fewer cells in the S and G2/M phases compared with the $\alpha_6\beta_4^{pos}$ CD71^{pos} cell fraction. These data demonstrate that the putative stem-cell fraction $\alpha_6\beta_4^{pos}$ CD71^{neg} is quiescent, in contrast with the rapidly proliferating $\alpha_6\beta_4^{pos}$ CD71^{pos} cell fraction, as described in skin epithelia (4). Another important attribute of keratinocyte stem cells is that they have the highest colony-forming ability among keratinocyte populations. In this study, the clonogenicity of the $\alpha_6\beta_4^{pos}$ CD71^{neg} cell fraction was confirmed, as it consistently produced significantly higher numbers of colonies than either the $\alpha_6\beta_4^{pos}$ CD71^{pos} cell fraction or the $\alpha_6\beta_4^{neg}$ cell fraction. This suggests that the $\alpha_6\beta_4^{pos}$ CD71^{neg} subpopulation has a much higher growth potential than the other two cell fractions.

Previous work (32) (33) has shown that keratinocyte stem cells have the smallest cell size among keratinocyte subpopulations. In our study, the $\alpha_6\beta_4^{pos}$ CD71^{neg} cell fraction had the highest colony numbers but the smallest cell size among the three cell populations. By contrast, the $\alpha_6\beta_4^{neg}$ cell fraction formed the fewest colonies and had the largest cell size. Also, the colony-forming ability was inversely correlated with cell size in the separated fractions, with colony-forming efficiency decreasing with increasing size of cells.

In the present study we have demonstrated that oral basal keratinocytes with the phenotype $\alpha_6\beta_4^{pos}$ CD71^{neg} have important stem-cell attributes. The physical ability to isolate OKSCs involves several important implications in defining more clearly the role of these cells in oral epidermal differentiation, and in studies of oral epithelial disorders, including carcinogenesis. We have shown that OKSCs can be successfully isolated using a magnetic

cell-sorting system. Magnetic cell separation offers the benefits of simplicity of operation and low cost, together with the high specificity and sensitivity provided by the use of immunospecific reagents. Transfer of this new approach to clinics may lead to significant improvement in developing well-organized oral human epithelia for use in intra-oral interventions.

Acknowledgements

This research was supported by the grant-in-aid no. 203905380001 from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

References

1. Papini S, Cecchetti D, Campani D *et al*. Isolation and clonal analysis of human epidermal keratinocyte stem cells in long-term culture. *Stem Cells* 2003;**21:** 481–494.
2. Alonso L, Fuchs E. Stem cells of the skin epithelium. *PNAS* 2003;**100:**11830–11835.
3. Watt F. Role of integrins in regulating epidermal adhesion growth and differentiation. *EMBO J* 2002;**21:**3919–3926.
4. Kaur P, Li A. Adhesive properties of human basal epidermal cells: an analysis of keratinocyte stem cells, transit amplifying cells, and postmitotic differentiating. *Cells J Invest Dermatol* 2000;**114:**413–420.
5. Webb A, Li A, Kaur P. Location and phenotype of human adult keratinocyte stem cells of the skin. *Differentiation* 2004;**72:**387–395.
6. Matsunaga T, Takahashi M, Yoshino T, Kuhara M, Takeyama H. Magnetic separation of $CD14^+$ cells using antibody binding with protein A expressed on bacterial magnetic particles for generating dendritic cells. *Biochem Biophys Res Commun* 2006;**350:**1019–1025.
7. Said T, Agarwal A, Zborowski M, Grunewald S, Glander H, Paasch U. Utility of magnetic cell separation as a molecular sperm preparation technique. *J Androl* 2008;**29:**134–142.
8. Blanpain C, Horsley V, Fuchs E. Epithelial stem cells: turning over new leaves. *Cell* 2007;**128:**445–458.
9. Miltenyi S, Muller W, Weichel W, Radbruch A. High gradient magnetic cell separation with MACS. *Cytometry* 1990;**11:** 231–238.
10. Powles R, Mehta J, Kulkarni S *et al*. Allogeneic blood and bone-marrow stem-cell transplantation in haematological malignant diseases: a randomised trial. *Lancet* 2000;**355:**1231–1237.
11. Weissman IL. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* 2000;**287:** 1442–1446.
12. Abts H, Emmerich M, Miltenyi S, Radbruch A, Tesch H. CD20 positive human B lymphocytes separated with the magnetic cell sorter (MACS) can be induced to proliferation and antibody secretion *in vitro*. *J Immunol Methods* 1989;**125:**19–28.
13. Manyonda IT, Soltys AJ, Hay FC. A critical evaluation of the magnetic cell sorter and its use in the positive and negative selection of CD45RO+ cells. *J Immunol Methods* 1992;**149:**1–10.
14. Li A, Pouliot N, Redvers R, Kaur P. Extensive tissue-regenerative capacity of neonatal human keratinocyte stem cells and their progeny. *J Clin Invest* 2004; **113:**390–400.
15. Dunnwald M, Tomanek-Chalkley A, Alexandrunas D, Fishbaugh J, Bickenbach JR. Isolating a pure population of epidermal stem cells for use in tissue engineering. *Exp Dermatol* 2001;**10:**45–54.
16. Kaur P, Li A, Redvers R, Bertoncello I. Keratinocyte stem cell assays: an evolving science. *J Investig Dermatol Symp Proc* 2004;**9:**238–247.
17. Romano A, Espana E, Yoo S, Budak M, Wolosin J, Tseng S. Different cell sizes in human limbal and central corneal basal epithelia measured by confocal microscopy and flow cytometry. *Invest Ophthalmol Vis Sci* 2003;**44:**5125–5129.
18. Zhou J, Jia L, Yang Y, Peng S, Cao Y, Duan E. Enrichment and characterization of mouse putative epidermal stem cells. *Cell Biol Int* 2004;**28:**523–529.
19. Croagh D, Phillips W, Redvers R, Thomas R, Kaur P. Identification of candidate murine esophageal stem cells using a combination of cell kinetic studies and cell surface markers. *Stem Cells* 2007;**25:**313–318.
20. Li A, Simmons P, Kaur P. Identification and isolation of candidate human keratinocyte stem cells based on cell surface phenotype. *Proc Natl Acad Sci U S A* 1998;**95:**3902–3907.
21. Morasso M, Canic M. Epidermal stem cells: the cradle of epidermal determination, differentiation and wound healing. *Biol Cell* 2005;**97:**173–183.
22. Yu Y, Flint A, Dvorin E, Bischoff J. AC133-2, a novel isoform of human AC133 stem cell antigen. *J Biol Chem*. 2002;**277:**20711–20716.
23. Trempus CS, Morris R, Bortner C *et al*. Enrichment for living murine keratinocytes from the hair follicle bulge with the surface marker CD34. *J Invest Dermatol* 2003;**120:**501–511.
24. Lowell S, Jones P, Le Roux I, Dunne J, Watt F. Stimulation of human epidermal differentiation by Delta–Notch signaling at the boundaries of stem-cell clusters. *Curr Biol* 2000;**10:**491–500.
25. Gandarillas A, Watt F. c-Myc promotes differentiation of human epidermal stem cells. *Genes Dev* 1997;**11:**2869–2882.
26. Zanet J, Pibre S, Jacquet C, Ramirez A, Alboran I, Gandarillas A. Endogenous Myc controls mammalian epidermal cell size, hyperproliferation, endoreplication and stem cell amplification. *J Cell Sci* 2005;**118:**1693–1704.
27. Pellegrini G, Dellambra E, Golisano O *et al*. p63 identifies keratinocyte stem cells. *PNAS* 2001;**98:**3156–3161.
28. McKeon F. p63 and the epithelial stem cell: more than status quo? *Genes Dev* 2004;**18:**465–469.
29. Michel M, Torok N, Godbout MJ *et al*. Keratin 19 as a biochemical marker of skin stem cells *in vivo* and *in vitro*: keratin 19 expressing cells are differentially localized in function of anatomic sites, and their number varies with donor age and culture stage. *J Cell Sci* 1996;**109:**1017–1028.
30. Fuchs E. Epidermal differentiation: the bare essentials. *J Cell Biol* 1990;**111:** 2807–2814.
31. Watt F, Phil D. Involucrin and other markers of keratinocyte terminal differentiation. *J Invest Dermatol* 1983;**81:** 100–1003.
32. Barrandon Y, Green H. Cell size as a determinant of the clone-forming ability of human keratinocytes Proc. *Proc Natl Acad Sci U S A* 1985;**82:**5390–5394.
33. Wan H, Stone MG, Simpson C *et al*. Desmosomal proteins, including desmoglein 3, serve as novel negative markers for epidermal stem cell-containing population of keratinocytes. *J Cell Sci* 2003;**116:** 4239–4248.