Human keratinocytes derived from the bulge region of hair follicles are refractory to differentiation

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Abstract. Human keratinocyte strains derived from the bulge region of plucked human follicles were successfully established from all 43 donors (age 24-76) regardless of the age and gender. The total cell number, number of population doublings and population doubling time were similar among the strains. These bulge-derived keratinocytes, BDKs, expressed keratin family genes specific to basal cell layers of the epidermis. They also expressed *CD34*, one of the bulge stem cell marker genes. The growth behavior and positivity of *CD34* indicate that BDKs contain stem cells. BDKs were cultured until confluency or treated with $CaCl_2$ to induce differentiation. Morphology and expression of keratin family genes in BDKs before and after differentiation induction with $CaCl_2$ were similar to those of epidermal keratinocytes obtained from skin biopsies (NHEKs). However, expression levels of *keratin-10*, a prickle cell layer marker, in $CaCl_2$-treated BDKs were lower than those in $CaCl_2$-treated NHEKs. Higher expression of *integrin-α6*, a basal cell layer marker, was also noted in BDKs than in NHEKs after differentiation induction. Expression of stem cell marker genes other than *CD34*, including *CD200, Sox2* and *NANOG*, was about the same at confluency in both cells, but significantly higher in BDKs than NHEKs after differentiation. These results indicate that BDKs were more refractory to differentiation than NHEKs. We then examined Wnt signaling inhibitor genes, *DKK-3* and *WIF-1* that function as tumor suppressors. *DKK-3* expression decreased in both BDKs and NHEKs after $CaCl_2$-induced differentiation. Expression of *WIF-1* decreased 50% in BDKs one day after $CaCl_2$ treatment and remained low, but was induced 1.7 times in NHEKs one day after $CaCl_2$ treatment and further induced thereafter (>2.5 times), suggesting that *WIF-1* may be involved in maintaining the differentiation-refractory status of BDKs. Since cancer stem cells in the skin have been reported to be similar to bulge-derived stem cells, our BDK strains may be of use in studying characteristics of cancer stem cells of the epidermis.

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Introduction

Epidermal stem cells were identified in the bulge region of hair follicles as label-retaining cells in mice, and their possible roles in skin carcinogenesis have been reported (1,2). The mouse bulge region has been noted as a reservoir of multipotent stem cells that differentiate to epidermal keratinocytes and other skin appendices, such as sebaceous glands, hairs, and fat cells, and also cells of other lineage, such as neuronal cells, blood vessels and mesenchymal lineage cells (3-8).

Keratin-15 (K-15) has been noted as a marker of mouse and human bulge stem cells (9). CD34- and K-15-positive cells are label-retaining, characteristic of stem cells. Higher integlin-α6 (ITG-α6) expression is also noted in undifferentiated keratinocytes than in differentiated keratinocytes. CD34-positive cells show higher ITG-α6 expression than CD34-negative cells (10), showing that CD34 is one of the markers of bulge stem cells. The CD34-positive cells in the bulge region, separated by FACS, are round and relatively small in size, and grow rapidly in culture (10). CD34-positive and K-15-negative cells, present in the bulge region and considered to be more undifferentiated than CD34-positive and K-15-positive cells, can differentiate to neuronal cells and blood vessels (5,6). These data indicate that the CD34-positive cells existing in bulge regions are multipotent stem cells.

Human adult stem cells isolated from hair follicles, and neural crest and neuron stem cells express *NANOG* and

OCT4 (Oct3/4). *Oct3/4*-positive cells were located in K-15-positive area of the bulge region *in vivo* (11). Analysis of gene expression in K-15-positive bulge stem cells by cDNA microarray identify several bulge stem cell-specific genes, such as *CD200, DKK-3, WIF-1, PHLDA1, follistatin, frizzled homolog 1, Sox2, Nanog* and *Oct-4* (8,12,13). In humans, CD200 has been reported to be a stem cell marker more specific than CD34 (8). Among stem cell marker genes shown above, *Oct3/4* and *SOX2* were used to establish human iPS cells (14).

Undifferentiated keratinocytes in the basal cell layer of the epidermis divide and differentiate to the keratinocytes in prickle, granular, and keratinized cell layers. $CaCl_2$ induces differentiation of epidermal keratinocytes in culture (15), with decreased expression of integlins-α6 and -ß1 and Keratins-5 and -14 (K-5 and K-14), markers of the basal cell layers, and increased expression of Keratins-1 and -10 (K-1 and K-10), markers of the prickle cell layers (5,16-18). However, there have been only a few reports on gene expression profiles of human bulge-derived keratinocytes in culture in relation to differentiation (8).

Bulge stem cells have been reported to play important roles in wound healing and skin cancer development. Implantation of mouse follicle cells into wound lesions of the skin result in recruitment of these follicle cells to cover wound lesions, but they are eliminated and replaced by basal cell layer-derived keratinocyte after the completion of wound healing, showing that follicle cells were not necessary for maintenance of the normal skin (19). However, molecular mechanisms of follicle cells in wound healing are not clear.

Basal cell carcinomas have been suggested to originate from the root sheath and/or the bulge region (20). CD34-positive cells in mouse bulge regions were found to proliferate during multi-stage skin carcinogenesis (21). In the CD34-knockout mice, basal cell carcinoma of the skin was not induced by treatment with chemical carcinogens (22), suggesting that CD34 is important to maintain cell proliferation and cancer formation. Recently, mouse early epidermal cancer cells were identified, which are similar to normal bulge stem cells phenotypically and functionally (23). These data suggest that the bulge region might be the reservoir of cancer stem cells as well as normal stem cells.

Skin biopsies are generally used to generate human epidermal keratinocyte cultures, but skin biopsy is highly invasive for donors. Plucked hair follicles can serve as an alternative source for epidermal keratinocytes in humans (24-26). In this study, we first established strains of human bulge-derived keratinocytes using plucked adult hair follicles and analyzed gene expression during $CaCl_2$-induced differentiation in comparison to neonatal skin epidermis keratinocytes (NHEKs) derived from skin biopsy.

Materials and methods

Subjects. Adult 43 volunteers, 16 females of 24-55 years of age, and 27 males of 25-76 years of age, were enrolled in this study under informed consent. There was no significant difference in the average age between the female (39.6±5.2 years) and male (39.5±6.1 years) groups (p=0.97). Twenty of 43 donors (age 37.1±6.3 years) had atopic features or atopic history including asthma and atopic dermatitis. This study was approved by the ethics board of Hyogo College of Medicine.

Primary cell culture from hair follicles. Hair follicles were plucked from occipital and temporal regions of each donor using forceps. Each hair was cut and the root portion containing hair follicles with the bulge region (~5 mm long) was incubated in Dispase (Godo Shusei Co., Ltd., Tokyo, Japan) (500 U/ml in DMEM, Gibco/Invitrogen Co., NY, USA) at room temperature for 5 min, placed in a 18 mm-well (Becton-Dickinson Labware, NJ, USA) with 150 μl of DKSFM medium (Gibco/Invitrogen), and incubated at 37˚C under 5% CO_2. The medium was renewed every day.

Primary-outgrowth (PO) was observed usually within 7 days of culture. On day 14, the number of follicles with PO was determined and expressed as the percentage of the total follicles inoculated (PO rate). When cells growing from PO occupied nearly half of the well surface (day 10-14), cells were rinsed with trypsin-EDTA solution [0.05% trypsin (Difco Lab, MI, USA) and 0.53 mM EDTA (Gibco/BRL, MD, USA) in PBS(-)] twice, and incubated for 2 min at room temperature. Cells were then treated with trypsin inhibitor solution (Kurabo Industries Ltd., Osaka, Japan), gently suspended by pipeting, and centrifuged. Cell pellets were resuspended in DKSFM and transferred into a 60-mm culture plate (Becton-Dickinson) (passage 1), and incubated with renewal of medium every 3 days. When cells reached 70% of confluency, they were trypsinized and $5x10^4$ cells were transferred into a culture plate. Viable cells were counted to calculate the number and time of population doublings.

Storage of hair follicles before primary culture. Hair follicles were put into a 1.5 ml cryotube (Iwaki, Tokyo, Japan) containing 1 ml of DKSFM medium with or without HEPES (20 mM, Gibco/Invitrogen), and kept at RT or 4˚C for 6 h with gentle shaking on a rocker platform (10 rpm) before use.

Skin biopsy-derived keratinocytes. Primary cultures of neonatal foreskin-derived epidermal keratinocytes (NHEKs) in the first passage were obtained from Kurabo (Kurabo Industries). Five NHEK strains from different lots were used. Approximately 5000 cells were incubated in a 60 mm culture plate (passage 2) with DKSFM medium without HEPES. Cells were subcultured in the same manner as bulge-derived cells.

Induction of differentiation. Bulge-derived cells and NHEKs in 3-4 passages were placed in 100 mm plates ($1x10^5$ cells/plate), and medium was renewed after two days. After 24-h incubation, cells reached in log-phase (day 0), and 1.2 mM $CaCl_2$ at a final concentration (Otsuka Pharm. Co., Ltd., Tokyo, Japan) was added. Medium was renewed on day 3.

RT-PCR. Isolation of total RNA and cDNA synthesis have been described (27). RT-PCR was performed using primers shown in Table I. PCR products were electrophoresed in agarose gels and the intensity of bands was measured by Adobe Photoshop CS2 (Adobe Systems, CA, USA). Gene expression levels were normalized by *GAPDH*. Real-time PCR was performed using Thermal Cycler Dice Real-Time

Table I. Primers and PCR condition used for RT-PCR and real-time PCR.

Gene	Forward primer	Reverse primer	A.T. (°C)[a]	Cycle
Keratin 5 (K-5)	5'-GAC GTG GAG ATC GCC ACT TAC C-3'	5'-AAA TTT GAC GCT GGA GCT GCT AC-3'	63	27
Keratin 14 (K-14)	5'-GTG ACC ATG CAG AAC CTC AAC GA-3'	5'-ACT CAT GCG CAG GTT CAA CTC TG-3'	63	27
Keratin 1 (K-1)	5'-AGA TCT CCA ACT TGC AGC AGT CCA-3'	5'-GGC TTG TGC TCA CAG ACA CAC TC-3'	63	27
Keratin 10 (K-10)	5'-TGA CCG CCT GGC TTC CTA CT-3'	5'-TCT GGC GCA GAG CTA CCT CA-3'	63	27
Integrin α6 (ITG-α6)	5'-GCG AGC CTT CAT TGA TGT GA-3'	5'-TCA GAT GGC TGA GCA TGG AT-3'	63	27
Integrin β1 (ITG-β1)	5'-CAG CAA CGG ACA GAT CTG CA-3'	5'-GAA CCA ACA GTC GTC AAC ATC-3'	63	27
CD200	5'-TCT CAC CCA AAT GGG ACC AC-3'	5'-AGG GCT CTC GGT CCT GAT TC-3'	63	33
CD34: first PCR	5'-GCA TCT GCC TGG AGC AAA AT-3'	5'-TGC AGC TGC ATG TGC AGA CT-3'	62	20
CD34: nested PCR	5'-AGG TAT GCT CCC TGC TCC TT-3'	5'-AGC TCC AGC CTT TCT CCT GT-3'	65	Real-time
Keratin 15 (K-15)	5'-AAG AAG TGG CCT CCA ACA CA-3'	5'-TCT CGG CCA GTG AGT TCT CC-3'	63	Real-time
DKK-3	5'-ACA GTG AGT GCT GTG GAG A-3'	5'-TCT CTG GAA GGC ACA GCA CA-3'	63	Real-time
WIF-1	5'-TGT CAA GAA GGT TGG CAT GG-3'	5'-TTA AGT GAA GGC GTG TGC TG-3'	64	Real-time
ATP5F1	5'-GCA GAA CAT GAT GCG TCG AA-3'	5'-TGC ACT TGG CAA TTG TCT CC-3'	63	Real-time

[a]A.T, annealing temperature.

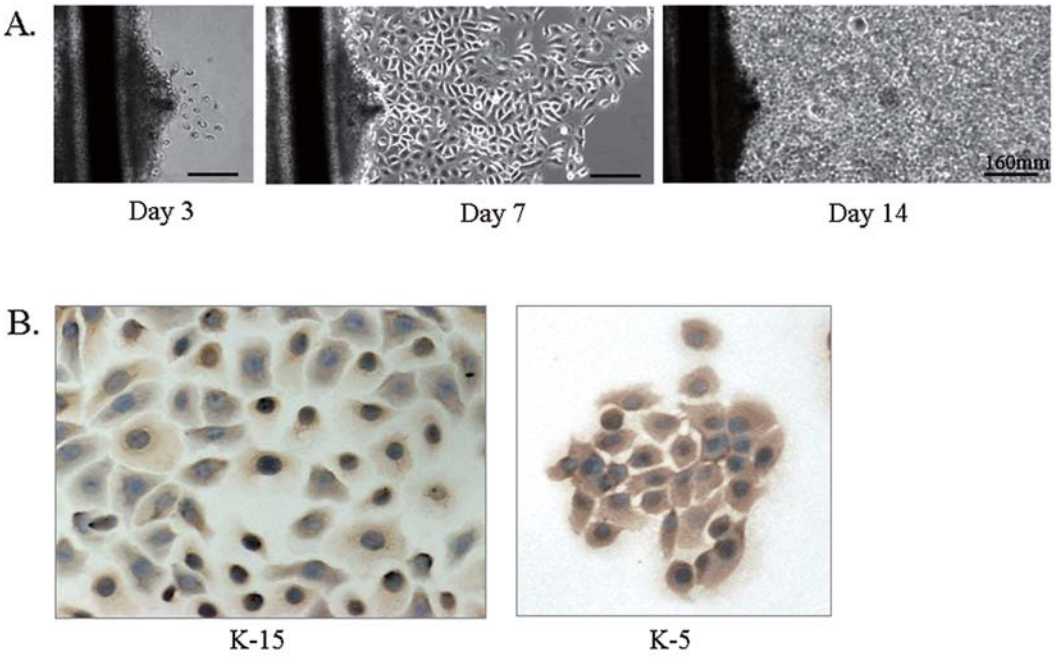

Figure 1. Morphology of primary outgrowth of a hair follicle. (A) Primary outgrowth cells from the bulge region of a hair follicle. Follicles were treated with Dispase, and incubated in an 18 mm-well with DKSFM medium at 37˚C (see Materials and methods). On day 3, ~20 cells were observed around the bulge region (primary outgrowth). On day 7, cells were cobble stone-like in morphology. On day 14, cells which were ~5000 were trypsinized, and transferred into a plate (passage 1). (B) Cells in passage 1 were immunostained with antibodies against K-15 (a bulge cell marker) and K-5 (a basal cell layer marker). All cells were K-15- and K-5-positive.

System (Takara Bio Inc., Otsu, Japan) and gene expression levels were normalized by *ATP5F1*.

Immunostaining. Cells were fixed with paraformaldehyde. Monoclonal antibodies against CD34, K-5, K-10, and K-15 (Novocastra Lab. Ltd., UK) were used as the first and anti-mouse IgG1-HP (Nichirei Biosciences Inc., Tokyo, Japan) as second antibodies.

Results

Primary outgrowth. Three to 20 hairs were plucked from occipital and temporal regions of each donor, and hair follicles containing the bulge region were immediately inoculated in a well with DKSFM. After 3-7 days of incubation, ~20 cells of cobble stone-like morphology were generated per bulge (primary-outgrowth, PO) (Fig. 1A). K-15, a bulge cell marker,

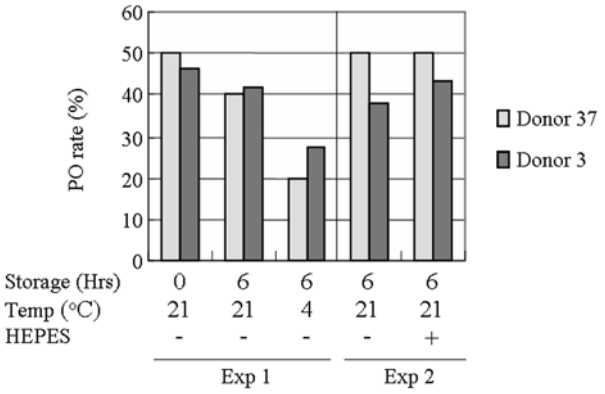

Figure 2. Storage condition after hair plucking and primary outgrowth rate. Hair follicles obtained from two donors (donor 37, a 26-year male and donor 3, a 55-year female) were soaked in DKSFM medium, transferred into a cryotube containing 1 ml of DKSFM, and kept at room temperature or at 4˚C before culture for 6 h with gentle shaking (exp. 1). The specimens were placed in culture wells. Primary-outgrowth (PO) was observed under a microscope on day 14 and the PO rate was calculated. The bulges were also kept in the DKSFM supplemented with or without HEPES (20 mM) for 6 h with gentle shaking at room temperature before culture (exp. 2). Five to ten follicles were used for each experiment.

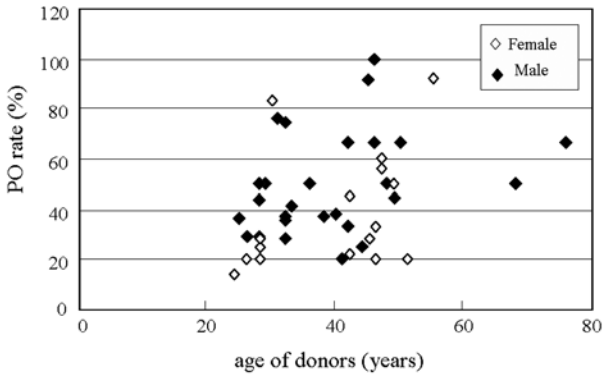

Figure 3. Primary outgrowth rate of all donors. BDK strains were established from 500 follicles from 43 donors. There were no significant differences in PO rate between male and female donors (p=0.15). There were no significant differences in age groups of every 10 years (p=0.07).

and K-5, a basal cell layer marker, were positive in >90% of PO cells (Fig. 1B). The number of bulge-derived PO cells reached ~5000 after 10-14 days of incubation with forming a stratified structure (Fig. 1A). These cells, termed bulge-derived keratinocytes (BDKs), were also K-5 and K-15 positive (data not shown), but negative for CD34.

The effect of storage of hair follicles after hair plucking was examined using hair follicles from two donors (donor 37, a 26-year-old male, and donor 3, a 55-year-old female). The PO rate after 6 h of storage at room temperature was about the same as that of fresh used follicles immediately after hair plucking (Fig. 2). The PO rate decreased 50% after 6 h of storage at 4˚C. The addition of HEPES to the storage medium had little effect on the PO rate (Fig. 2). Based on these results, extracted hair follicles were immediately placed in DKSFM medium without HEPES, and used for culture within 6 h.

The PO rate of 500 follicles obtained from 43 donors (24-76 years of age) ranged from 14 to 100% (average: 46.5%) (Fig. 3). There was no significant difference in the PO rate between males and females (49.0±5.2% vs 38.7±5.9%, p=0.15), or among different age groups of every 10 years (p=0.07). The PO rate in atopic donors did not differ significantly from that in non-atopic donors (40.7±9.9% vs 49.8±5.9%, p=0.07). PO was observed in two follicles out of three obtained from a 76-year-old male with alopecia areata, and the PO rate of a 66-year-old male was ~50% (Fig. 3).

Cell growth and gene expression of BDK cells in sequential passages. Approximately 5000 cells were obtained from a follicle on day 14 of primary culture. These cells originated from each follicle (cell strain) were cultured separately. From five donors aged 26-66, more than five BDK strains were established. From each donor, five BDK strains were randomly selected and analyzed for cell growth. All 25 BDK strains from five donors showed similar growth. Growth curves of cells from two donors, aged 66 and 26, are shown in Fig. 4A. All BDK strains could be cultured until 7-9 passages, and the number of population doublings was between 25 and 27. Between passages 2 and 4, the average population doubling time was 26.1 h (Table II). Total yields of BDKs from a follicle were $4.3x10^{11}$ on average (Table II). These data showed that cell growth characteristics of BDK strains were similar and independent of the age of donor.

Five strains of NHEKs were used to study growth curves. Five thousand NHEKs were inoculated in a plate for the first passage. Growth curves of all NHEK strains were similar (Fig. 4A). The number of population doublings was less in BDKs than in NHEKs. The population doubling time between passages 2-4 was longer in BDKs than NHEKs. Total yield of BDKs was ~1/10 of NHEKs (Table II).

Three BDK strains obtained from three donors, a 26-year-old male, a 66-year-old male, and a 55-year-old female, and three NHEK strains were used for gene expression studies. In both BDKs and NHEKs, *CD34* was detected in passages 2-4 (Fig. 4B) but hardly detectable at passage 4. *K-5*, a basal cell layer marker, was positive until passage 7. *K-10*, a prickle cell layer marker gene, was negative initially, but became positive in NHEKs and weakly positive in BDKs after passage 6. These results showed that, *CD34*-positive cells existed in cultured BDKs and NHEKs at least until passage 4. Spontaneous differentiation occurred after passage 4 but this process was slower in BDKs than NHEKs.

Comparison of gene expression between NHEK and BDKs. Treatment of BDKs with 1.2 mM $CaCl_2$ caused flattening of cells and arrest of growth in 3 days. After 7 days of $CaCl_2$ treatment, flattened and stratified cells with expression of K-10 were observed by immunostaining (Fig. 5). The growth arrest and morphological changes of BDKs after $CaCl_2$ treatment were similar to those of NHEKs.

Gene expression was examined after treatment with $CaCl_2$ in BDKs and NHEKs. *K-5* and *K-14* genes were expressed and $CaCl_2$ treatment caused no significant changes in expression levels (Fig. 6A). In the absence of $CaCl_2$, *K-1* and *K-10* were hardly detected in NHEKs and BDKs on

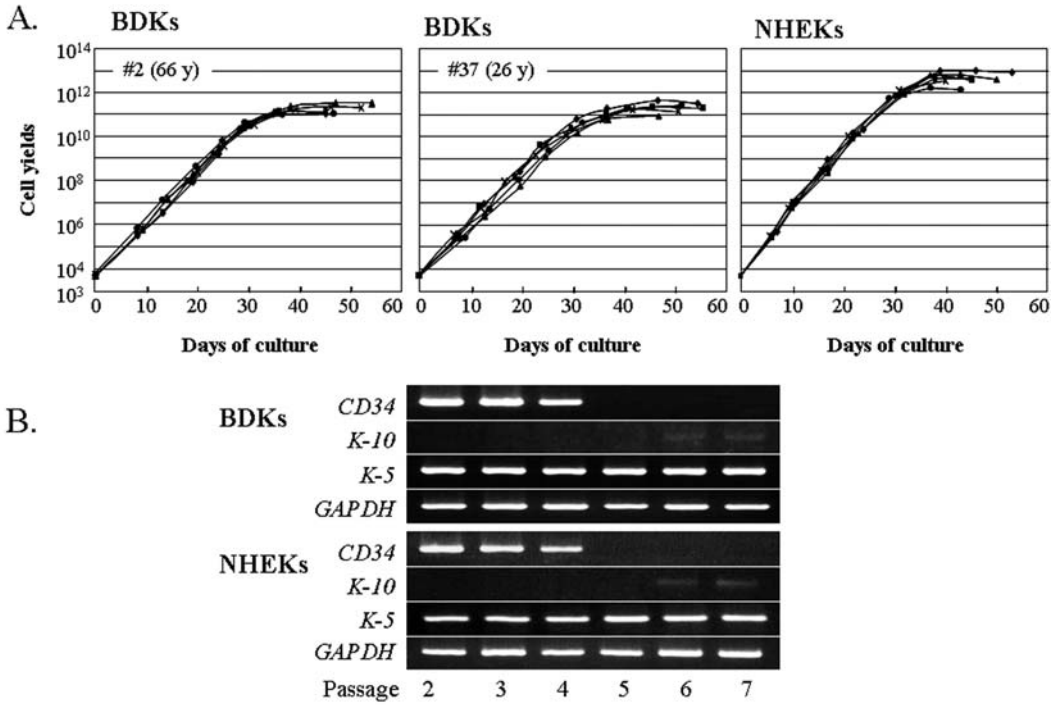

Figure 4. Cell growth and expression of the CD34, K-10 and K-5 genes. (A) Growth curves of BDK strains obtained from a 66-year-old male and a 26-year-old male are shown. From each donor, more than five BDK strains were obtained and five strains were randomly selected for growth assay. For NHEKs, five strains were used. At passage 1, all BDKs, ~5000, obtained from primary outgrowth were inoculated into a plate. Five thousand NHEKs in passage 1 were also inoculated in a plate. When BDKs and NHEKs reached 70% of confluency, cells were trypsinized and subcultured (passage 2). The points on the growth curves indicate passages. After passage 7, cells became flattened and large, resembling senescent cells. (B) Expression of the *CD34, K-10* and *K-5* genes during passages was examined. Cells in a log-phase culture were harvested and gene expression was examined by RT-PCR (see Materials and methods). *GAPDH* was used for normalization of the expression level.

Table II. Number of population doublings (PDs), PD time and total cell yield of BDKs and NHEKs.

BDKs Donor no.	Age	No. of PDs	PD time (h)[a]	Total cell yield (x10^{11})
2	68	26.5±1.0	27.5±1.7	4.5±3.1
3	55	26.3±1.0	28.3±0.5	4.1±2.1
12	46	25.5±1.0	30.3±0.5	1.7±0.9
26	33	27.0±1.4	28.7±1.6	9.4±8.5
37	28	25.5±1.0	27.4±0.6	1.8±2.6
Av.		26.1±1.1	28.3±1.0	4.3±6.9
NHEKs strain no.	**Age**	**No. of PDs**	**PD time (h)[a]**	**Total cell yield (x10^{11})**
1	0	31	25.1	110
2	0	30	26.3	74
3	0	29	25.1	49
4	0	30	25.3	71
5	0	28	25.5	10
Av.		29.6±1.1	25.5±0.5	65±34

From each donor, five BDK strains established from five follicles were used for cell growth studies. Approximately 5000 BDKs from primary outgrowth of each follicle were inoculated into a culture plate at passage 1. Five NHEK strains were used and 5000 cells were also inoculated at passage 1. Number of population doublings (PDs), PD time and total cell yield of each BDK and NHEK strains were calculated from growth curves. [a]Average time (hours) from 2 to 4 passages.

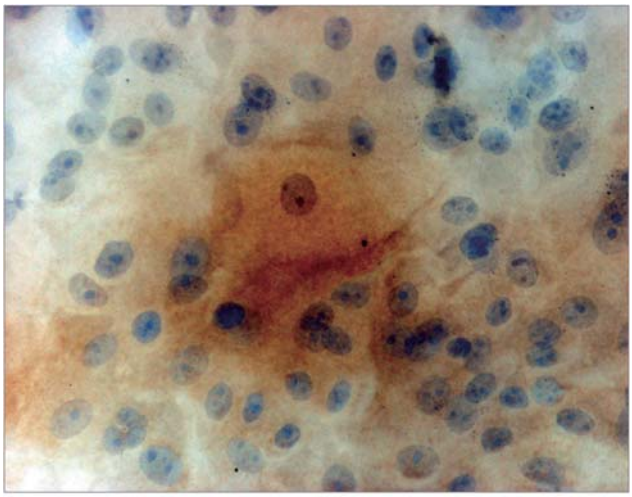

Figure 5. Expression of K-10 in BDKs after differentiation. Cells in passage 4 were inoculated in a plate and incubated for two days, then treated with $CaCl_2$ (1.2 mM) for 7 days. Immunostaining with antibodies against K-10 (a prickle cell layer marker) showed flattened and stratified cells were K-10 positive.

days 0 and 1, became detectable on day 3 in NHEKs and on day 5 in BDKs and their expression levels further increased on day 7. In NHEKs with $CaCl_2$ treatment, *K-1* and *K-10* became detectable on days 1 and 3 in BDKs. But levels of

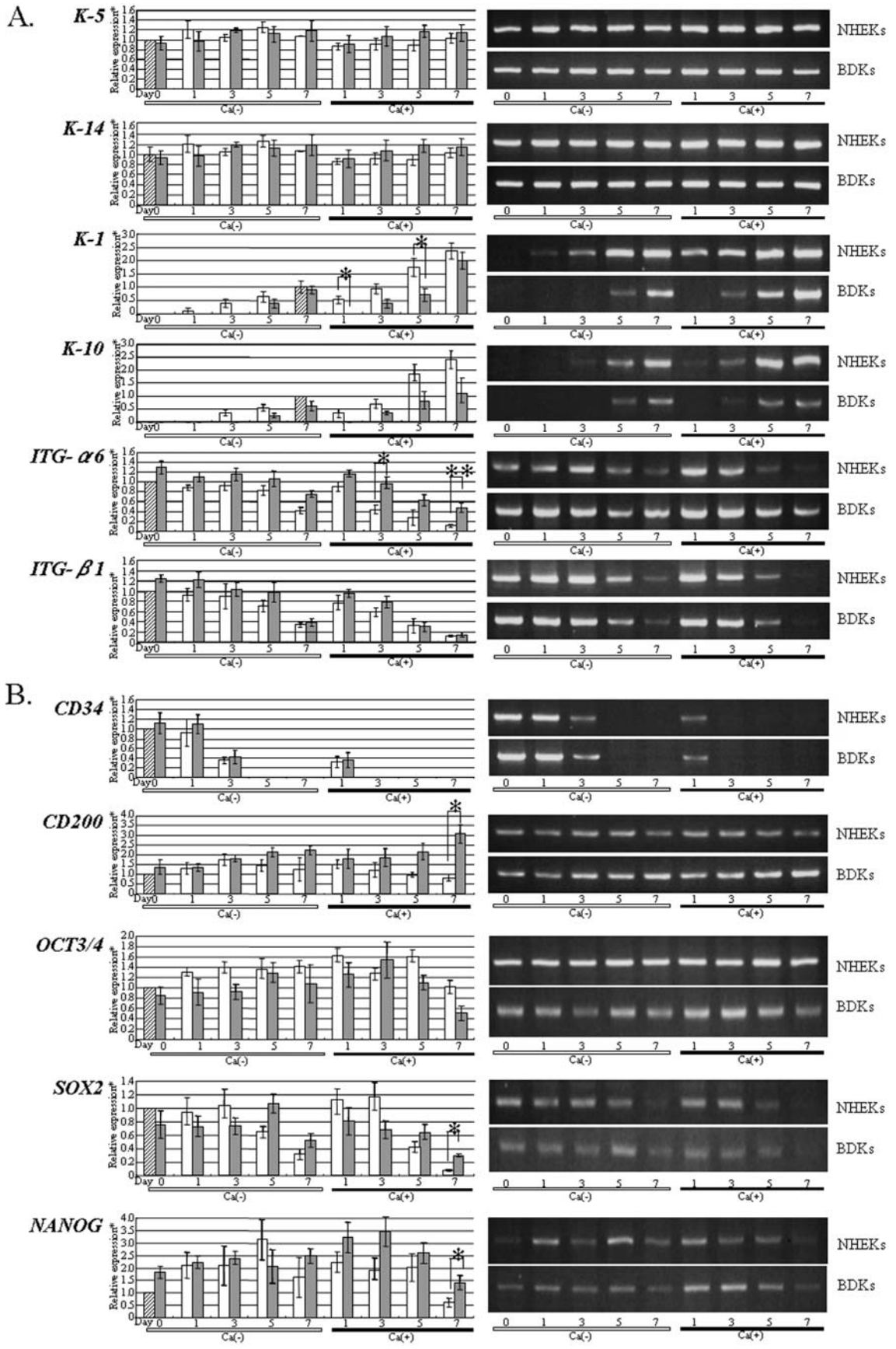

Figure 6. Expression of keratinocyte-specific genes and stem cell markers in BDKs and NHEKs during differentiation. Cells in passage 2-4, inoculated in a culture plate, incubated for two days and medium was renewed (day 0). Cells were incubated with or without $CaCl_2$ (1.2 mM). Cells were harvested on days 1, 3, 5 and 7, and analyzed for *Keratins(K)-5, -14, -1* and *-10, integrins (ITG)-a6 and -β1, CD34, CD200, OCT3/4, SOX2* and *NANOG* genes by RT-PCR (see Materials and methods). White and gray columns indicate NHEKs and BDKs, respectively. Expression levels were normalized to the levels of *GAPDH*. Expression levels of NHEKs on day 0 were set as 1.0 (shaded columns) except *K-1* and *-10*. For *K-1* and *K-10*, expression levels of NHEKs on day 7 were set as 1.0 (shaded columns). Each column shows the average of three independent experiments. Bar, I SD. (A) Expression of epidermal keratinocyte-specific genes. *K-5, K-14, ITG-a6* and *ITG-β1* are basal cell layer markers, whereas *K-1* and *K-10* are prickle cell layer markers. (B) Expression of stem cell marker genes. $^{*}p \leq 0.05$. $^{**}p \leq 0.01$.

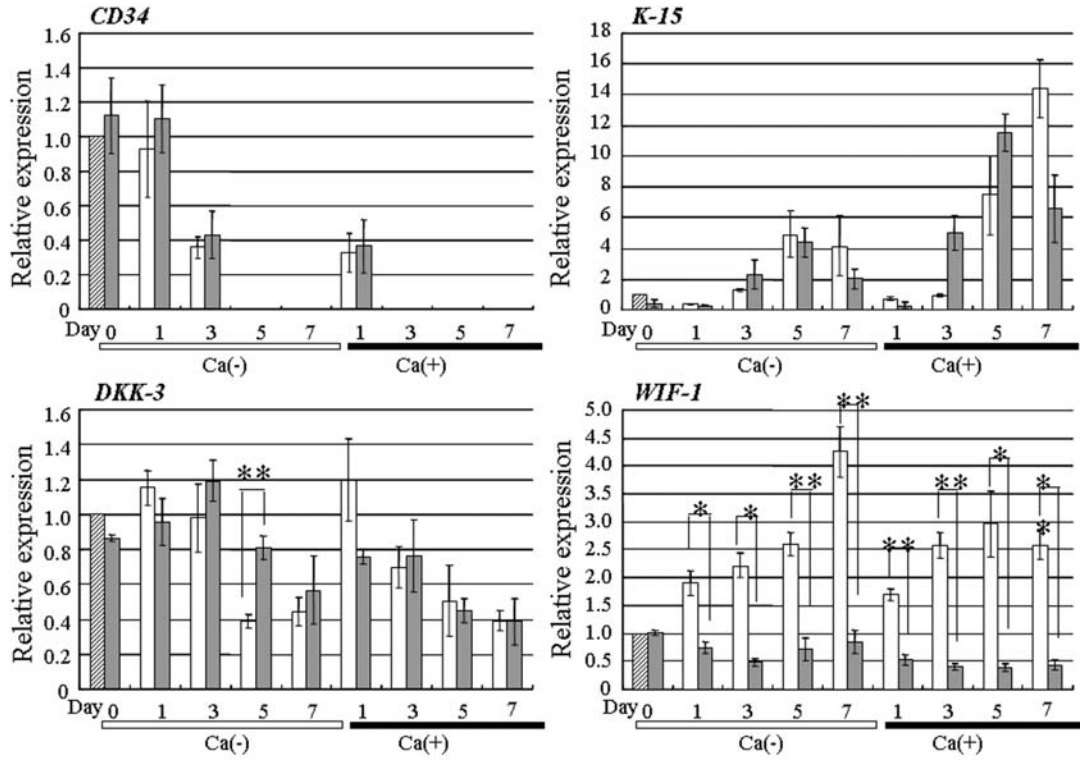

Figure 7. Real-time PCR analysis of *CD34, K-15, DKK-3* and *WIF-1* expression in BDKs and NHEKs. Expression levels of *CD34* and *K-15*, reported as bulge stem cell markers, and *DKK-3* and *WIF-1*, Wnt signaling inhibitors, were normalized to the levels of *ATP5F1*. Expression levels in NHEKs on day 0 were set as 1.0 (shaded columns). White and gray columns indicate NHEKs and BDKs, respectively. Each column shows the average of three independent experiments. Bar, I SD. $^{*}p\leq0.05$. $^{**}p\leq0.01$.

both genes were lower in BDKs than NHEKs (Fig. 6A). The expression levels of *ITG-α6* on day 7 with $CaCl_2$ treatment was significantly higher in BDKs than in NHEKs ($p<0.01$) (Fig. 6A). These results showed that changes of expression of prickle cell layer marker genes were smaller in BDKs than NHEKs.

We examined five stem cell markers, *CD34, CD200, Oct3/4, SOX2*, and *NANOG*, in BDKs and NHEKs after $CaCl_2$ treatment. Without $CaCl_2$ treatment, expression of *CD34* was positive until day 3 and then negative on days 5 and 7 in both BDKs and NHEKs (Fig. 6B). With $CaCl_2$ treatment, *CD34* was expressed on day 1 only in BDKs and NHEKs (Fig. 6B). Expression of *CD200*, reported as a more specific bulge stem cell marker than CD34, was positive in NHEKs on day 0 and the expression levels were stable with or without $CaCl_2$ until day 7. In BDKs, the expression levels of *CD200* were similar to those of NHEKs without $CaCl_2$ until day 7 and with $CaCl_2$ until day 5. But on day 7 with $CaCl_2$, significantly higher expression of *CD200* was observed in BDKs than in NHEKs ($p<0.05$). *OCT3/4* levels were similar in both BDKs and NHEKs without $CaCl_2$ treatment. In the presence of $CaCl_2$, expression of *OCT3/4* was slightly increased until day 5 but decreased on day 7 in both cells (Fig. 6B). The expression of *SOX2* decreased on day 7 without $CaCl_2$ treatment in both cells. In the presence of $CaCl_2$, *SOX2* levels in NHEKs was significantly lower than those in BDKs on day 7 ($p<0.01$). *NANOG* levels showed some increase during incubation in the absence of $CaCl_2$ in both BDKs and NHEKs. In the presence of $CaCl_2$, *NANOG* levels increased until day 5 and then decreased on day 7 in both cells with expression levels in NHEKs slightly lower than in BDKs ($p=0.05$). These data suggest that BDKs may be refractory to differentiation.

We then examined expression levels of *CD34* and *K-15* using real-time PCR. In both BDKs and NHEKs, *CD34* expression decreased rapidly to be essentially undetectable on day 5 (Fig. 7). *K-15* expression was low in both cells in the absence of $CaCl_2$ on days 0 and 1, and significantly increased on days 3-7. In the presence of $CaCl_2$, *K-15* levels were highly elevated on days 3-7 (Fig. 7). These results indicate that *K-15* was one of the differentiation markers of the BDKs and NHEKs, and not suitable to detect bulge-stem cells in culture.

We also examined *DKK-3* and *WIF-1* genes involved in the Wnt signaling pathway. In the absence of $CaCl_2$, *DKK-3* levels were similar in NHEKs and BDKs except day 5 when NHEKs showed significantly lower levels than BDKs ($p<0.01$). $CaCl_2$ treatment suppressed *DKK-3* expression to a similar extent in both cell types. In the absence of $CaCl_2$, *WIF-1* expression remained low in BDKs, but in NHEKs *WIF-1* levels were elevated 2-fold on day 1 and 4-fold on day 7, resulting in higher *WIF-1* expression levels in NHEKs than in BDKs ($p<0.05$ on days 1 and 3, $p<0.01$ on days 5 and 7). Similarly, in the presence of $CaCl_2$, *WIF-1* expression became 50% in BDKs on day 1 and remained low until day 7, while in NHEKs, *WIF-1* expression level was 2- to 3-fold on

days 1-7, resulting in much higher *WIF-1* expression levels in NHEKs ($p<0.01$ on days 1, 3 and 7, $p<0.05$ on day 5).

Discussion

Primary culture and characteristics of BDK cells. We report the cultivation of BDKs not using invasive skin biopsies but from hairs plucked from donors. With the culture method used, hair specimens from all 43 donors yielded BDKs with an average PO rate of 47%, regardless of age and gender of the donor. BDKs were also obtained from atopic donors with the average PO rate of 41%, indicating that BDKs can be established from donors with variety of backgrounds. Interestingly, three hairs of a 76-year old subject with alopecia areata also yielded BDKs with the number of population doublings, the population doubling time, the total cell yields, and morphology of this donor's BDKs similar to those of younger donor's BDKs.

The expression of *CD34*, one of the stem cell markers, was positively detected in cells in primary outgrowth and in early passages (Fig. 4B). However, immunostaining failed to detect CD34-positive cells in the primary outgrowth, suggesting low levels of CD34 expression and/or very few CD34-positive cells in the primary outgrowth. Only 1-2% of cells separated directly from human bulges have been reported to be CD34-positive by FACS analysis (25). Positive *CD34*, high proliferation ability, and age-independent growth of BDKs strongly suggest that BDKs are derived from the stem cell fraction existing in bulge regions.

From one hair follicle, $2x10^{11}$-$9x10^{11}$ BDKs were obtained and the life span in culture was at least 3 months (Fig. 3 and Table II). Hence, total cell yield was $4.0x10^{7}$-$1.8x10^{8}$ times the number of cells in passage 1. In comparison of growth between BDKs with NHEKs, we found BDKs showed less total cell yield and fewer number of population doublings than NHEKs. If CD34-positive cells constitute 1-2% cells in a bulge as reported (25), only 50-100 stem cells might exist in ~5000 BDKs in primary outgrowth. Since 50-100 cells divided at least 6 times to reach 5000 cells, 5000 cells used for the first passage were already in six population doublings. Consequently, the number of population doublings and other cell proliferation ability of BDKs may be the same or better than NHEKs, supporting the possibility that our BDKs were derived from bulge stem cells.

Gene expression of BDKs during differentiation. After induction of differentiation with $CaCl_2$, the reduction of CD34 expression was observed in both NHEKs and BDKs in a similar manner. Morphological changes and decreased expression of *K-5*, one of the basal cell markers, were also observed in both cell types. However, lower induction of *K-1* and *K-10*, prickle cell layer markers, and smaller reduction of expression of *ITG-α6*, a basal cell layer marker, were detected in BDKs than in NHEKs (Fig. 6A). Expression of *K-15*, reported as a bulge-stem cell marker, was significantly induced in both NHEKs and BDKs after $CaCl_2$ treatment (Figs. 6A and 7). Immunostaining also showed that higher expression of K-15 in both cells after $CaCl_2$ treatment (data not shown). We analyzed *CD200* in BDKs which has been reported to be a more specific bulge stem cell marker than CD34 (8). *CD200* levels in BDKs were found to be higher after $CaCl_2$ treatment (Fig. 6B), suggesting that *CD200*, like *K-15*, was not a suitable marker for bulge stem cells, at least in our culture condition. *CD200* was selected as one of bulge stem cell marker genes by cDNA microarray analysis of K-15-positive cells in the bulge region that were isolated from skin biopsy specimens by microdissection, trypsinization, and FACS sorting (8). Since these procedures may alter characteristics of cells, gene expression profiles in such cells may differ from those in BDKs obtained by our culture method which might preserve more physiological conditions.

We also analyzed several other genes reported as specific to bulge stem cells. Expression of *SOX2* and *NANOG* was higher in BDKs than in NHEKs after $CaCl_2$-induced differentiation (Fig. 6B), suggesting that BDKs are more resistant to differentiation than NHEKs.

Recently, active Wnt signaling has been considered to be important to maintain embryonic and adult stem cells and cancer stem cells (23,28). Wnt signaling is also active in basal cell carcinomas of the skin (29). Since high expression of two Wnt signaling-inhibitor genes, *DKK-3* and *WIF-1*, has been detected in bulge stem cells (8), we examined expression of these genes in BDKs and NHEKs. Lower expression of *DKK-3*, also called *REIC*, is detected in many human cancers than in normal tissues (30,31). Reduced *DKK-3* expression occurred as an early event of tumor development (32), and its overexpression induced apoptosis in tumor cells (33). Expression of *WIF-1*, wnt inhibitory factor-1, that directly binds Wnt proteins to inhibit Wnt signaling, was low in several solid tumors (34,35).

DKK-3 was expressed in NHEKs and BDKs but expression levels decreased to half on day 5 after $CaCl_2$ treatment in both cells (Fig. 7). *WIF-1* expression was about the same in BDKs and NHEKs on day 0 and $CaCl_2$ treatment increased 2-fold in NHEKs and decreased to half in BDKs on day 1. On day 3, expression levels further increased in NHEKs, but remained low in BDKs (Fig. 7), indicating that regulatory mechanisms of *WIF-1* may be different between NHEKs and BDKs. It has been proposed that, in cancer cells, levels of *WIF-1* are negatively regulated through hypermethylation in the promoter regions (34,35). The possibility exists that methylation may be lost in NHEKs but maintained or induced in BDKs during differentiation.

Our results strongly suggest that BDKs are refractory to differentiation. BDKs have also been reported to play a role in wound healing (19) and development of psoriasis and cancer (36). Our data suggest that maintenance of active Wnt signaling pathway in BDKs through suppression of *WIF-1* expression may be important for immediate response of BDKs to skin injury facilitating re-epithelization and for keeping undifferentiated features of BDKs that may be important to develop basal cell carcinoma of the skin. Our data also suggest that characteristics of BDKs, such as expression of stem cell markers, high proliferation capacity, and resistance to differentiation, are related to down-regulation of *WIF-1* expression. Constitutional activation of Wnt signaling pathway in bulge stem cells may lead to carcinogenesis. Activation of *WIF-1* expression may be a key for prevention of skin cancers originating from the bulge region.

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