Desmosomal proteins, including desmoglein 3, serve as novel negative markers for epidermal stem cell-containing population of keratinocytes

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Summary

No single method has been universally adopted for identifying and isolating epidermal stem/progenitor cells, and the emergence of new markers of stem cell populations is worth exploring. Here we report, for the first time, that clusters of basal keratinocytes at the tips of the rete ridges in human palm, previously recognised as a major repository of stem cells, had very low levels of desmoplakin protein and mRNA expression, compared with cells at the sides of the ridges or above the dermal papillae. We found that in populations of palm keratinocytes, selected by their ability to adhere rapidly to type IV collagen, there were significantly reduced levels of desmoplakin and other major desmosome proteins. We then showed that a low desmoglein 3 (Dsg3) expression on the cell surface could be used to enrich for a cell population with high clonogenecity, colony forming efficiency and enhanced proliferative potential, but with a low ability to form the abortive clones, compared with populations with a higher Dsg3 expression. Moreover, stringent sorting of populations showing both β1 integrin-bright and Dsg3-dull expression enabled even further enrichment of a population containing the putative epidermal stem cells. These findings provide the basis for a new strategy for epidermal stem/progenitor cell enrichment, and encourage further study of the role of desmosomes in stem cell biology.

Key words: Desmoplakin, Desmoglein 3, Desmosomes, Stem cells, Keratinocytes

Introduction

Desmosomes are intercellular junctions expressed abundantly in all epithelia including the epidermis. The principal constitutive molecules of these junctions include two family members of the desmosomal cadherins, desmogleins (Dsgs) and desmocollins (Dscs), and a collection of cytoplasmic plaque proteins including desmoplakin (Dp), plakoglobin (Pg) and plakophilins (Pkps) (Garrod, 1993; Garrod et al., 2002a; Garrod et al., 2002b). Previous studies, by electron microscopy and morphometry, revealed that desmosome expression increases as keratinocytes mature and migrate from basal into suprabasal layers of skin (White and Gohari, 1984; Wan et al., 2003).

Skin homeostasis is governed by a small subpopulation of keratinocyte stem cells (Potten and Morris, 1988; Lavker and Sun, 2000; Watt, 2001). It has been known for some years that basal keratinocytes are not homogeneous and possess diverse cell populations including stem cells, transit amplifying cells and post-mitotic cells undergoing differentiation (Potten and Morris, 1988; Jones and Watt, 1993; Jones et al., 1995; Dunnwald et al., 2001). Owing to the slow cycling nature of epidermal stem cells in vivo, conventional approaches to determine stem cell location have relied on cell kinetic analyses using either bromodeoxyuridine (BrdU) or [^{3}H]thymidine to follow up the label-retaining cells within the tissue. Thus, the location of stem cells in a variety of epithelia has been determined in vivo (Lavker and Sun, 1983; Cotsarelis et al., 1989; Taylor et al., 2000). For instance, stem cells in monkey palm were identified at the tips of deep rete ridges by pulse-labelling with [^{3}H]thymidine (Lavker and Sun, 1983).

Cultured human keratinocytes, however, may not maintain all their in vivo characteristics after removal from their 'niche' or microenvironment but are clonogenic (Potten and Morris, 1988; Jones and Watt, 1993; Jones et al., 1995) and able to generate three distinct clonal forms owing to the varied self-renewal capacities of individual keratinocytes (Barrandon and Green, 1987). Holoclones, founded mainly by stem cells, appear large with a smooth circular perimeter and have the highest reproductive capacity. Paraclones, founded by cells undergoing terminal differentiation, appear small and are known as abortive clones. Meroclones are intermediate between holoclones and paraclones in their morphology and behaviour. It has been suggested that analysis of colony formation and frequency of clone types after a period of growth in vitro predicts the intrinsic nature and capacity of the proliferative potential of the original keratinocyte populations (Barrandon and Green, 1987).

Growing interest has focused on the role that stem cells play in homeostasis, wound repair and tumorigenesis (Tu et al., 2002) as well as on their identification and use as a therapeutic

option in tissue bioengineering, gene therapy and for the treatment of a variety of diseases (Rama et al., 2001). Considering the potential ethical issue of using embryonic stem cells, identifying multiple markers to isolate pure keratinocyte stem cell populations from adults is of value. It is important both for understanding the basis of stem cell science and also for stem-cell-mediated clinical replacement therapy. However, studies of stem cell properties have been hampered by the lack of appropriate surface markers that could facilitate their identification and isolation. Many attempts have been made to identify putative markers of epidermal stem cells (Jones and Watt, 1993; Jones et al., 1995; Li et al., 1998; Dunnwald et al., 2001), one of which is the β1 integrin (Jones and Watt, 1993; Jones et al., 1995), the positive cell surface marker. It has been shown that the basal keratinocytes with a high colony forming efficiency (CFE) have the highest β1 integrin staining characteristics (Jones and Watt, 1993; Jones et al., 1995). Other studies suggest that a keratinocyte population containing stem cells can be isolated according to high levels of integrin α_6 expression and low levels of transferrin receptor (Li et al., 1998; Tani et al., 2000). However, negative markers alone are also considered useful for isolating the putative stem cell populations. For example, gap junction protein connexin 43 has been proposed to be a negative marker for epidermal stem cells (Matic et al., 1997; Matic et al., 2002). Several molecular signal pathways such as NF-κB, Wnt/β-catenin, Sonic hedgehog/patched and delta/notch have been described in the literature as being involved in the control of the stem cell compartment (Watt, 2001).

Based on the evidence that desmosomes become more numerous as keratinocytes differentiate and mature, it seemed reasonable to hypothesise that desmosomes may appear sparse in the epidermal stem/progenitor cells. In this study we report, for the first time, that this indeed is the case. We found that the epidermal basal keratinocytes, either in vitro or in vivo, show heterogeneity in desmosome expression. Clusters of cells with low Dp expression can be identified at the tips of deep rete ridges in palm skin as well as within the colonies of cultured keratinocytes. Fractionated populations, enriched for β1-bright cells exhibit inversely low desmosomal protein expression. We show that sorting keratinocytes that express low levels of Dsg3 results in enrichment for a cell population that exhibits features in common with the epidermal stem cells, i.e. high clonogenecity, CFE and proliferative potential. Moreover, we show that sorting for β1 integrin-bright/Dsg3-dull keratinocytes enhances even further the enrichment of epidermal stem cells.

Materials and Methods

Antibodies

The mouse monoclonal and rabbit polyclonal antibodies (Ab) used were as follows: 115F, mouse Ab to Dp (a gift from Professor D. Garrod) (North et al., 1999); AHP320, rabbit Ab to Dp (Serotec); AHP321, rabbit Ab to Dsgs (Serotec) (Wheeler et al., 1991; Wan et al., 2003); AHP322, rabbit Ab to Dscs (Serotec); Dsc3-U114, mouse anti-human Dsc3 (RID, USA); 5H10, mouse Ab to the extracellular domain of Dsg3 (a gift from Dr M. Amagai) (Proby et al., 2000); PG5.1, mouse Ab to Pg (Cymbus) (Wan et al., 2003); PP1-5C2, mouse Ab to Pkp1 (Progen) (Wan et al., 2003); LP34, mouse conformational Ab to a broad spectrum of basal-cell-associated keratins (from Professor B Lane) (Jones and Watt, 1993); DH1, rabbit anti-involucrin Ab (gift from Cancer Research UK) (Dover and Watt, 1987); direct conjugate 12G10:Cy5, mouse anti-human β1 integrin recognising a ligand-induced binding site epitope (Mould et al., 1995) (a gift from Cancer Research UK); direct conjugate CD29:FITC (Serotec); β-actin Ab ab6276 (Abcam). Secondary Ab used were Alexa Fluor 488 or 568 conjugated goat anti-mouse or anti-rabbit IgG, respectively (Molecular Probes via Cambridge BioSciences) and mouse IgG:RPE (DAKO). Zenon Alexa Fluor 647 mouse IgG_1 labelling kit (Molecular Probes via Cambridge BioSciences).

Immunofluorescence for adult palm skin section and cultured keratinocytes

Normal palm and non-palm (mainly breast) skins were obtained under approval from St Thomas' Hospital's ethics committee. Cryosections of skin and the cultured keratinocytes were processed and labelled as described previously (Wan et al., 2003).

Human primary keratinocyte culture

The adult human keratinocyte cultures were generated following a standard procedure (Navsaria, 1994) and as described previously (Wan et al., 2003).

Laser scanning confocal microscopy

Dual immunofluorescence images were acquired following the procedure described previously (Wan et al., 2003).

In situ hybridisation

In situ hybridisations for Dp mRNA on cryosections of palm skin and cultured keratinocytes were performed following the protocol provided by the manufacturer (BIOGNOSTICK, Germany) as detailed elsewhere (Wan et al., 2003).

Immunoblotting

Protein extracts and western blotting for the major desmosomal proteins were carried out as described previously (Wan et al., 2003).

Adhesion to type IV collagen

This procedure was carried out as previously described (Jones and Watt, 1993; Jones et al., 1995). Briefly, the plastic dishes were coated overnight at 4°C with 100 μg/ml collagen (Sigma), and then incubated with 0.5 mg/ml heat-denatured bovine serum albumin (BSA) (Sigma) at 37°C for 1 hour and washed in serum-free medium before use. The suspended cells were harvested using trypsin-EDTA and allowed to attach to the substrate for 20 minutes (rapid adhesion). The unattached cells were then transferred to a fresh dish and allowed to attach for 24 hours (slow adhesion). After 24 hours, the unattached cells were removed and fresh medium was added. Cells plated into the dish without adhesion separation were used as the control. Cells were cultured for a further 8 hours before protein extraction or fixation for immunofluorescent labelling.

Immunofluorescent labelling and flow cytometry

Immunofluorescent labelling and flow cytometry were carried out as described previously (Jones and Watt, 1993). Briefly, for fluorescence-activated cell sorting (FACS) analysis, passage 1 keratinocytes were suspended in 0.25% trypsin containing 1 mM calcium chloride in PBS and filtered through a 70 μm nylon mesh (Falcon). For single labelling, cells were incubated on ice with a saturating concentration of Dsg3 Ab 5H10 or CD29:FITC for 5-10 minutes before washing in wash buffer [Dulbecco's modified Eagle's medium (Gibco) plus 0.1%

bovine serum albumin (BSA; Sigma)]. Primary antibody-labelled cells were incubated with Alexa Fluor 488-conjugated IgG for 5 minutes on ice. After washing thoroughly, cells were kept either in PBSABC plus 0.1% BSA and 0.1% NaN_3 for flow cytometry or in keratinocyte-culture medium containing 1% foetal bovine serum (FBS) for FACS. Propidium iodide (5 μg/ml) was added for viability gating. For dual labelling, cells were either labelled with 5H10-RPE or 5H10:Zenon Alexa Fluor 647 followed by CD29:FITC. Non-viable cells were gated out using DAPI.

Cells were sorted using a FACS Vantage (BD Biosciences, San Jose, CA) or a Moflo (DakoCytomation, Fort Collins, CO) sorter. The sorted cells were collected into tubes containing keratinocyte culture medium supplemented with 10% FBS before being plated at different cell densities on J2 feeder cells, which were treated with mitomycin C (Sigma) prior to use.

Colony analysis

Cells were grown on feeders for different time periods. The colonies were assessed by phase-contrast microscopy (Zeiss TELAVAL 31) and photographed (KYOCELA, Japan). At the end of each experimental time point, colonies in wells (n=3) were fixed with equal volumes of ice-cold methanol and acetone for 15 minutes, washed in PBS and stained either with 1% Rhodamine B (Sigma) and 1% Nile Blue (Sigma) (Jones and Watt, 1993) or with LP34 and DH1 then DAKO EnVision Doublestain System. The stained colonies were scanned and the images were analysed in Adobe Photoshop. The feeder background was masked with white (255 in greyscale) to avoid its influence before analysis of the colonies in software OPTILAB PRO 2.6.1 (GRAFTEK, France). Parameters of colony density per dish and colony number and area (size) were acquired. The colony density was calculated as the percentage of each dish covered by colonies. Results are presented as mean±s.e.m. of triplicate samples. CFE was the ratio of colony number to plating cell number (Jones and Watt, 1993). The mean colony area was calculated from all the individual colonies and presented as the mean±s.e.m. Statistical analysis was conducted using one-way analysis of variance (ANOVA). Significant differences were determined at $P<0.05$.

Colony assessment and total cell number determination

To determine the frequency of abortive colonies, colonies were selected randomly and counted blindly using a phase-contrast microscope. Colonies were scored on their morphological feature (Jones and Watt, 1993; Jones et al., 1995). Total colony numbers after 2 weeks in culture were counted and total cell number was determined using a haemocytometer. Differences of clone type between groups were analysed by Chi-Square and differences were deemed statistically significant if $P<0.05$.

Viability assay

The FACS-sorted and control cells, and non-FACS-sorted control cells were plated at a series of titration densities in 96-well plates coated with 100 μg/ml collagen. The viability assay was performed following the protocol provided with CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega).

Results

Low desmosomal protein expression in the basal keratinocyte layer in palm skin

Using immunofluorescence and in situ hybridisation, we found heterogeneity in Dp and Dsg expression restricted to the basal layer of human adult palm in vivo (Fig. 1A,B). At the tips of the rete ridges, we observed a low level of Dp expression compared with keratinocytes, at the sides of the ridges or above the dermal papillae, and with those in the suprabasal layers within the rete ridges. Similarly, in the primary cultured adult palm keratinocytes, which resembled cells of the inner layers of the epidermis and contained a mixture of stem and transit amplifying cells, as well as post-mitotic keratinocytes undergoing differentiation, Dp expression varied (Fig. 1D,E). Clusters of cells, which were small and located within the colonies, showed a low Dp expression level which was restricted largely to the cell-cell junctional area (Fig. 1D arrow). In contrast, cells that were large and frequently located at the interior of the colonies expressed abundant Dp throughout the whole cell (Fig. 1D).

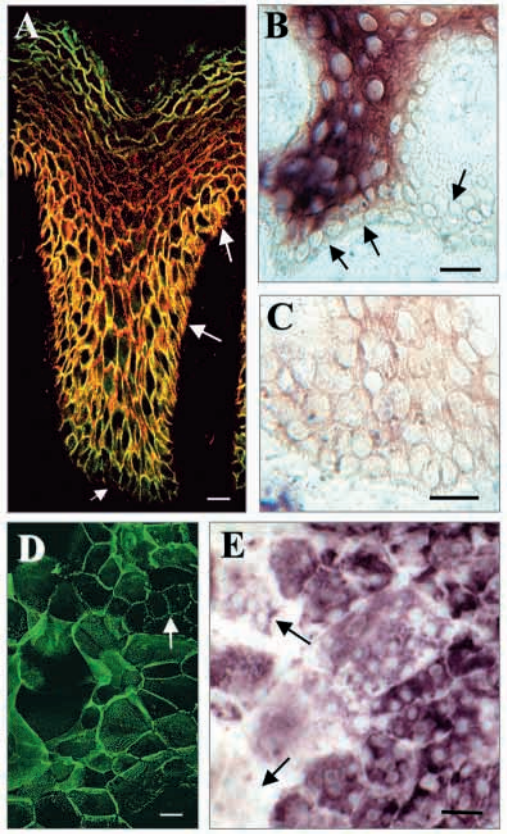


Fig. 1. Diversity of Dp expression in human palm keratinocytes both in vivo and in vitro. (A) Confocal microscopic image of human palm skin labelled for Dp (green) and Dsgs (red). Both proteins show a low level of expression at the tips of the epidermal ridge (arrow head) but abundant immunoreactivity at the side of the ridge or above the dermal papillae (arrows), as well as in the suprabasal layers. (B) In situ hybridisation showing Dp mRNA expression in palm skin, and (C) the negative control with the randomer. Note clusters of small rounded cells located at the tips of the palm ridge express little or low level of Dp mRNA compared with the suprabasal cells. (D) Immunofluorescent staining for Dp in cultured palm keratinocytes. Diversity of Dp expression is seen and a few small rounded cells show a very low level of Dp expression, which is largely restricted to the cell boundary (arrow). Larger cells show Dp staining throughout the cells. (E) In situ hybridisation showing Dp mRNA expression in cultured palm keratinocytes. Note heterogeneous expression of Dp mRNA in vitro and little or no expression in clusters of small cells (arrows). Scale bar: (A-D) 20 μm, (E) 50 μm.

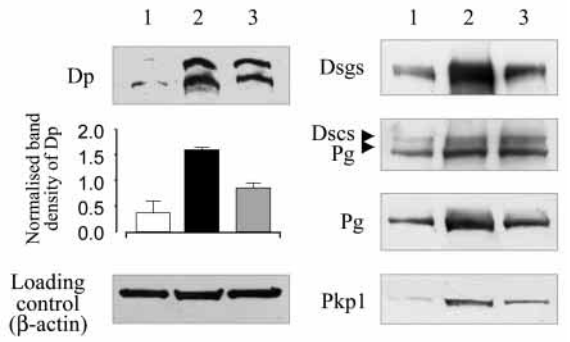


Fig. 2. Human adult palm keratinocyte populations, separated by rapid and slow adhesion to type IV collagen, show different desmosomal protein expression. Two cell populations, separated by rapid (<20 minutes, lane 1) or slow (>20 minutes-24 hours, lane 2) adhesion to type IV collagen, were analysed by western blotting for the desmosomal proteins Dp, Dsgs, Dscs, Pg and Pkp1. Non-separated cells were used as the control (lane 3). The graph shows densitometric analysis of eight blots from five experiments on Dp expression (mean±s.e.m.). Note Dp expression in the slowly adhering cell population is about fourfold that of the rapidly adhering cell population. Control cells show an intermediate level of expression as expected. Similar expression profiles were observed for the other desmosomal proteins. β-actin demonstrates equal loading of the protein samples.

High β1 integrin-expressing keratinocytes have low level of Dp expression

To test our hypothesis that the putative adult epidermal stem cells express fewer desmosomes we used an established protocol to enrich for keratinocytes that contain stem cells from human skin (Jones and Watt, 1993; Jones et al., 1995). We separated trypsin-dissociated adult palm keratinocytes into two populations by rapid (<20 minutes) and slow (>20 minutes to 24 hours) adhesion to type IV collagen, a selection procedure based on levels of functional β1 expression. Unfractionated cells were used as the control. Western blotting analyses (n=8) from a minimum of five experiments revealed that rapidly adhering cells appeared to have low Dp expression. In contrast, the slowly adhering cells showed about a fourfold increase in Dp expression, while an intermediate level of expression of this protein was seen in the control cells, as expected (Fig. 2). Other desmosome proteins, including Dsgs, Dscs, Pg and Pkp1 also showed qualitatively similar expression profiles when the rapidly and slowly adhering cell populations were compared (Fig. 2). To confirm that the rapidly adhering cells contained higher levels of the surface activated β1 integrin (Jones and Watt, 1993; Jones et al., 1995) we fixed the cells prior to direct immunofluorescent staining with an antibody recognising functional cell surface β1 integrin [12G10 (Mould et al., 1995) directly conjugated to Cy5]. We found that there was a significantly higher level of activated β1 integrin expression in the rapidly, rather than the slowly, adhering cells (data not shown). We also compared β1 integrin expression in these two populations by flow cytometry and found consistently higher surface β1 integrin levels in the rapidly, rather than slowly, adhering cells (data not shown), as previous reported (Jones and Watt, 1993). Immunofluorescent staining for Dp in these two cell populations showed relatively less punctate staining in the rapidly- as compared to the slowly- adhering cells although diversity of Dp expression was still seen within the rapidly adhering cells (data not shown), suggesting existence of keratinocytes heterogeneity. Taken together, these findings show that selecting palm keratinocytes, by rapid adhesion to a β1 integrin ligand (type IV collagen), enriches for a sub-population that has a low level of desmosomal protein expression including Dp and Dsgs.

Adult palm keratinocytes with a low Dsg3 level are capable of forming large, actively expanding colonies

The different expression level of desmosomal proteins between rapidly and slowly adhering cell populations suggested that desmosomes might serve as a negative marker for putative epidermal stem cell enrichment. In order to sort for the viable cells with low desmosome expression using FACS, antibodies directed against the extracellular domains of desmosomal cadherins in basal keratinocytes were required. We therefore tested two available antibodies, Dsc3-U114 (mouse antibody directed to the extracellualr anchor domain of human Dsc3) and 5H10 (mouse antibody directed against the amino-terminal EC1 and part of EC2 in the extracellular domain of Dsg3). We found that only 5H10 worked in flow cytometry and thus we developed an assay for detecting the cell surface desmosomal protein Dsg3. Palm keratinocytes at passage 1 were dissociated with 0.25% trypsin in the presence of 1 mM Ca^{2+} (Takeichi, 1977) and labelled with 5H10. Bound antibody was detected with goat anti-mouse IgG conjugated to Alexa Fluor 488 prior to FACS analysis (Fig. 3A). The viable keratinocytes were selected in the square box on the basis of their light-scattering characteristics. Dead cells were gated out with propidium iodide (less than 10% of the cells). The selected cells were further fractionated into two categories on the basis of Dsg3 expression. We sorted for 20% of cells with the lowest level of Dsg3 ($Dsg3^{dim}$) and for the 40% of cells with the highest level of Dsg3 ($Dsg3^{bri}$). Cells were then plated onto the feeder cells (J2 strain) at three different densities, and grown for 5, 7, 9, 11 (Fig. 3B) or 14 days. At each time point, colonies were fixed prior to staining for both keratins and involucrin, and visualised with EnVision Doublestain System (DAKO). Parameters of colony density (Fig. 3C), CFE (Fig. 3D) and colony size (Fig. 3E) were scored (see Materials and Methods). Overall, the $Dsg3^{dim}$ population developed higher colony densities and larger colony sizes than $Dsg3^{bri}$ cells at almost every time point measured. By day 11 both colony density and size were increased significantly compared with the $Dsg3^{bri}$ population (Fig. 3C,E). A slightly higher CFE was also observed in $Dsg3^{dim}$rather than $Dsg3^{bri}$ population at 11d (Fig. 3D). These results suggested that cells in the $Dsg3^{dim}$ population had greater growth ability and were capable of generating large, actively expanding colonies in short-term (2 weeks) culture.

$Dsg3^{dim}$ keratinocyte populations shows greater overall proliferative capacity and survive better than $Dsg3^{bri}$ cells in long-term culture

In order to investigate the longer-term culture of the adult keratinocytes sorted for either low- or high-Dsg3 levels, cells were monitored for multiple further passages until all the cells were completely exhausted. At each passage, cells were

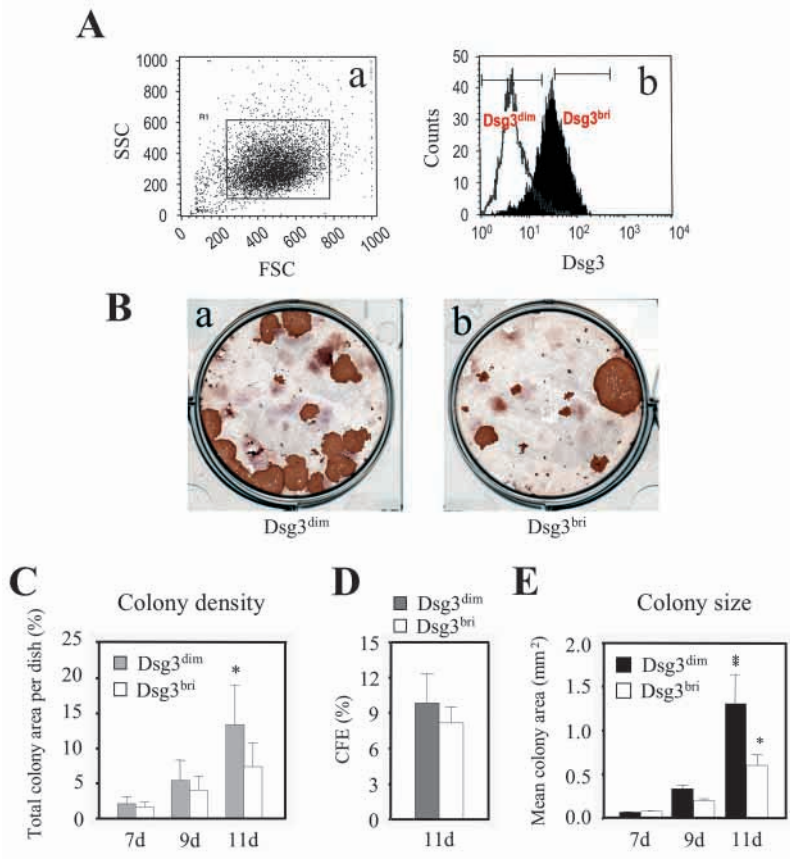
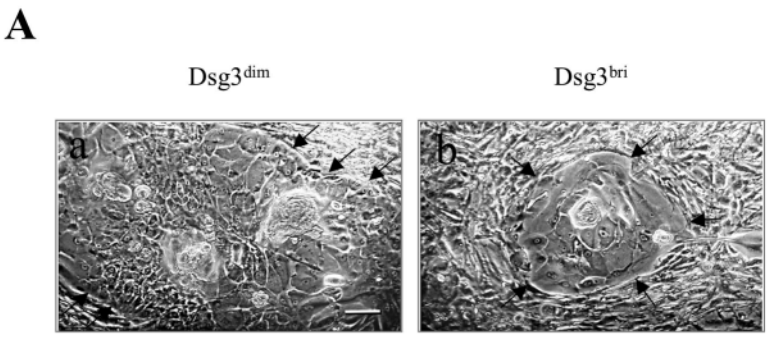


Fig. 3. The Dsg3^{dim} population of adult palm keratinocytes shows a higher CFE than the Dsg3^{bri} population. (A) Dot plot showing the FSC and SSC of a suspension of cultured keratinocytes (a). Each dot represents one cell, and both FSC and SSC are measured in arbitrary units on a linear scale. Cells were sorted into two groups: fluorescence dim (Dsg3^{dim}: 20% of the cells with the least Dsg3) and fluorescence bright (Dsg3^{bri}: 40% of the cells with the highest Dsg3) populations (b). Alexa Fluor 488-conjugated IgG was used as the negative control (unfilled). (B) The sorted cells were plated onto the J2 feeder cells at 400 cells per well, and grown for 11 days in culture. Colonies were stained for keratins and visualised with anti-mouse HRP. More colonies grew in the Dsg3^{dim} population (a) than in the Dsg3^{bri} population (b). (C) Colony density analysis, as indicated by total colony area per dish (i.e. percentage of total area of dish) between Dsg3^{dim} and Dsg3^{bri} populations (mean±s.e.m. of triplicate samples). There is a higher total colony area in the Dsg3^{dim}, than in the Dsg3^{bri}, population and by 11 days the total colony area per dish in the Dsg3^{dim} population is significantly larger (*P<0.05), nearly twofold that of Dsg3^{bri}. (D) CFE (ratio of colony number to plated cell number) at 11 days (mean±s.e.m. of triplicate). A slightly higher CFE is seen in Dsg3^{dim} than in the Dsg3^{bri} population. (E) Mean colony area (size) of Dsg3^{dim} and Dsg3^{bri} populations at three time points. The colony area increases with time and by 11 days the mean colony area in both Dsg3^{dim} and Dsg3^{bri} is significantly larger than that at 7 days or 9 days (*P<0.05). In particular that of Dsg3^{dim} is twofold that of Dsg3^{bri} by 11 days (average ~1.4 mm^2 vs ~0.6 mm^2, **P<0.05).

counted and plated at the same density. Cells formed heterogeneous colonies in the cultures, and we found that there were three major and distinct colonies in our study of the adult human keratinocytes. One resembled holoclones as described by Barrandon and Green (Barrandon and Green, 1987), and cells in this type of the colony were all very small and capable of generating large, actively growing colonies after 2 weeks. Another type of colony resembled the paraclones or abortive clones (Barrandon and Green, 1987; Jones and Watt, 1993; Jones et al., 1995) and was very small in size. Cells in this colony resembled the differentiating squamous cells and were almost all positive for involucrin (data not shown). The third type of colony showed intermediate features. In our study we therefore assessed the frequency of the abortive clones. We found that in older cell generations (4-14 weeks), differences in colony appearance between the two sorted populations were more apparent. Fig. 4 shows examples of the different colonies observed in Dsg3^{dim} and Dsg3^{bri} sorted cell cultures at an early passage number. Colonies in Dsg3^{bri} cultures were very small and mostly abortive within the first 4 weeks (passage 2). By passage 3 (5-6 weeks), very few or no colonies were found in the Dsg3^{bri} population. In contrast, in the Dsg3^{dim} population, there were still many large colonies observed from passage 2 (Fig. 4A,B), and the total cell output in this population was sixfold that of Dsg3^{bri} cells (30×10^5 Dsg3^{dim} versus 5×10^5 Dsg3^{bri}). Moreover, the Dsg3^{dim} cell population was capable of sustaining longer-term generation for a further 9-10 weeks and thus had remarkably longer culture duration than Dsg3^{bri} cells. Double staining the colonies for Dp and involucrin expression showed that a higher proportion of cells and colonies in the Dsg3^{bri} population were involucrin-positive compared with cells of the Dsg3^{dim} population, suggesting that the majority of cells in the Dsg3^{bri} population were terminally differentiating (data not shown). To assess the clone type quantitatively, we selected colonies randomly in each population in passage 2 and scored the abortive colonies under ‘blind’ conditions (Fig. 4B). The total number of colonies assessed in Fig. 4B reflected the frequency and population density of colonies in flasks. Of 96 colonies assessed for the Dsg3^{dim} population, only 59 (61.5%) were abortive colonies. In contrast, 32 out of 33 colonies (97.0%) in Dsg3^{bri} cells were abortive (χ^2: P<0.05). Furthermore, these data suggested that cells with low Dsg3 expression had a greater overall proliferative potential and were capable of expanding more and for a longer duration in culture.

To explore whether we were able to achieve similar results in keratinocytes derived from a site other than thick skin, we repeated the same studies on thin skin keratinocytes, in two separate experiments. Again, we found that keratinocytes from thin (mainly breast) skin sorted into low and high Dsg3 populations also showed significantly larger, more actively growing colonies but lower frequency of abortive colonies in the population with a low expression level of Dsg3, suggesting high clonogenecity and CFE in this population (data not shown). These data demonstrated that sorting keratinocytes from thin skin for low desmosome expression also enabled us to enrich for a cell population with some common features of the putative stem/progenitor cells. There is, therefore, no



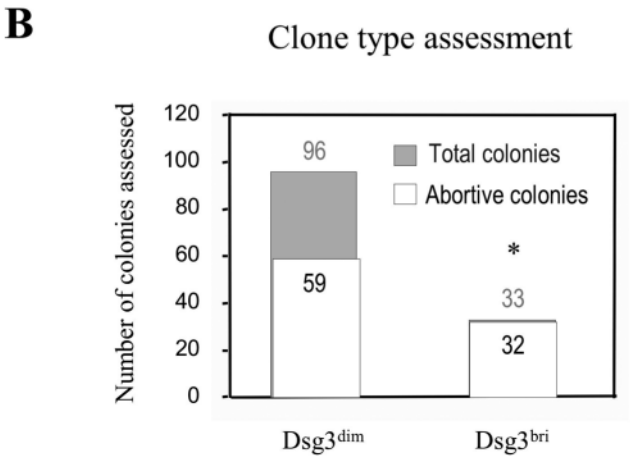


Fig. 4. Dsg3^{dim} population of palm keratinocytes survived better than Dsg3^{bri} population in long-term culture. (A) Phase-contrast images (a,b; arrows delineate margin of colony) of different clone types in Dsg3^{dim} and Dsg3^{bri} at passage 2. Large colonies are observed in the Dsg3^{dim} (a) but not the Dsg3^{bri} population, in which almost all the colonies are abortive (b). (B) Assessment of the frequency of abortive colonies in Dsg3^{dim} and Dsg3^{bri} at passage 2. Of 96 colonies in Dsg3^{dim} only 59 are abortive colonies, however, 32 out of 33 are abortive colonies in Dsg3^{bri} (P<0.05). Scale bar: (in A) 50 μm.

difference between thick and thin skin for these characteristics although they are known to differ in terms of desmosome composition and distribution (Wan et al., 2003).

To verify whether the FACS procedure affects the cell viability in each fraction, we also performed a cell proliferation assay (data not shown). We found that at each time point examined, for up to 5 days, there was no difference between the viability of fractionated cells compared with the control undergoing the FACS.

Combining the two surface markers of β1 integrin and Dsg3 to sort keratinocytes for a β1^{bri}/Dsg3^{dim} population enriches considerably the putative keratinocyte stem cell population

The β1 integrin has been proposed to be a useful cell surface marker for the enrichment of epidermal keratinocytes containing stem cells (Jones and Watt, 1993; Jones et al., 1995). However, the β1 integrin-bright population may contain both stem cells and transit amplifying cells (Potten and Morris, 1988; Jones and Watt, 1993; Jones et al., 1995; Li et al., 1998; Birkenbach and Dunnwald, 2002), implying that this method of sorting stem cells, although powerful, is not sufficient to identify the true stem cell compartment. We further enriched the colony-forming cell population by combining two surface markers, β1 integrin and Dsg3, to obtain a β1^{bri}/Dsg3^{dim} population (from either thick or thin skin). The dissociated keratinocytes (of passage 1) were dual labelled with 5H10 in conjunction with Zenon Alexa Fluor 647 and CD29:FITC. Dead cells were gated out with DAPI. We sorted for two cell populations, i.e. β1^{bri}/Dsg3^{dim}: the cells with the highest β1 and least Dsg3 constituting of 11.5% of the population, and β1^{bri}/Dsg3^{bri}: cells with the highest β1 and Dsg3 constituting 5.39% of the population (Fig. 5A). The β1-bright cells in this study included 15~25% of the total sorted cells (Jones and Watt, 1993). As colony-forming cells represent a more primitive population and showed low forward light scatter (FSC) and side light scatter (SSC) profiles (Jones and Watt, 1993), we investigated light characteristics of these two cell populations using an FSC vs SSC plot. The majority of cells (85%) in the β1^{bri}/Dsg3^{dim} population had lower SSC characteristics (Fig. 5Ab) than the β1^{bri}/Dsg3^{bri} population (40% only) (Fig. 5Ac), possibly suggesting that cells in β1^{bri}/Dsg3^{dim} population may be less mature. Colony forming assays in short-term culture showed more colonies in the β1^{bri}/Dsg3^{dim} population than the β1^{bri}/Dsg3^{bri} population (Fig. 5B,C). There was at least a fourfold increase of colony density and CFE in thick skin keratinocytes, and at least a threefold increase in colony density and CFE in thin skin keratinocytes in the β1^{bri}/Dsg3^{dim} populations (Fig. 5C). Similar results were consistently observed in three independent experiments and among two to three cell strains (cells from different individuals) in each experiment (n=7). Compared with the results of the colony density and the CFE data with a single Dsg3 marker (Fig. 3C,D), there was a remarkable increase of both colony density and CFE from combining the two markers together. Since kinetic studies suggest stem cells represent 10% of the basal keratinocyte population (Potten and Morris, 1988; Lavker and Sun, 2000; Watt, 2001), whereas sorting for a β1^{bri} population enriches for 40-50% of the cells (Dunnwald et al., 2001; Birkenbach and Dunnwald, 2002), our study clearly has identified a subpopulation in the β1^{bri} cells (i.e. those that are also Dsg3^{bri}) that did not exhibit stem cell characteristics and showed poor CFE (Fig. 5). Colonies with low Dp staining which were involucrin-negative were seen more frequently in β1^{bri}/Dsg3^{dim} than β1^{bri}/Dsg3^{bri} populations (data not shown). Furthermore, we found sustained and significantly increasing differences between these two populations especially in long-term cultures (up to 14 weeks). Cultivation of the β1^{bri}/Dsg3^{dim} cells showed much better cell survival and longer culture duration than cells in the β1^{bri}/Dsg3^{bri}population (average 14.3 weeks vs 4.2 weeks, Fig. 6). The total cell outputs in the β1^{bri}/Dsg3^{dim} population were approx. threefold higher at passage 1 (average 10.1×10^5 β1^{bri}/Dsg3^{dim} vs 2.8×10^5 β1^{bri}/Dsg3^{bri}) with greater differences in growth capacity from passage 2 onwards (14 weeks) since few or none of the cells from β1^{bri}/Dsg3^{bri} population were able to survive beyond passage 2 (4 weeks).

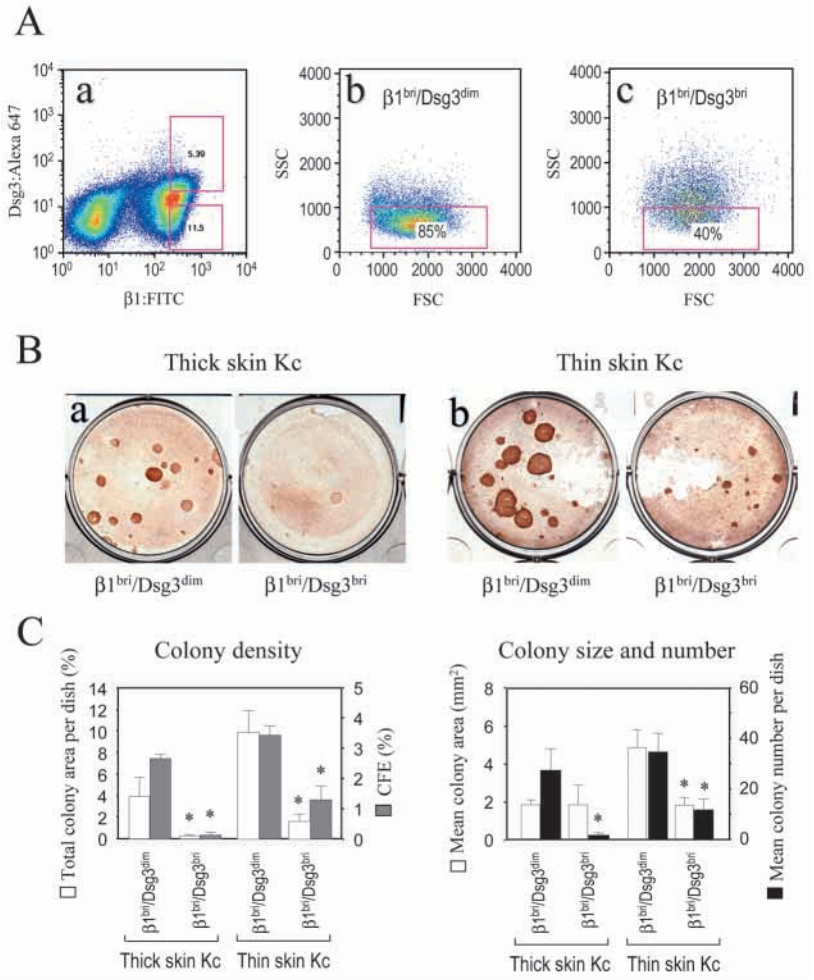


Fig. 5. Sorting keratinocytes for a $\beta1^{bri}/Dsg3^{dim}$ population further enhanced the enrichment of colony forming cells. (A) Keratinocytes dual labelled for β1 integrin and Dsg3 were sorted into two groups: $\beta1^{bri}/Dsg3^{dim}$ (11.5% of the cells, with the highest β1 and least Dsg3) and $\beta1^{bri}/Dsg3^{bri}$ (5.39% of the cells, with both the highest β1 and Dsg3) populations (a). (b,c) FSC and SSC plot of $\beta1^{bri}/Dsg3^{dim}$ and $\beta1^{bri}/Dsg3^{bri}$ populations, respectively. Note that 85% of cells in $\beta1^{bri}/Dsg3^{dim}$ (b) but only 40% of cells in $\beta1^{bri}/Dsg3^{bri}$ (c) have low SSC characteristics. Relatively more cells in c show high FSC and SSC characteristics. (B) Sorted sole (thick skin; a) and breast (thin skin; b) keratinocytes (Kc) were plated onto the feeders at 1000 or 500 cells per well, respectively, and grown for 11 days. Colonies were stained for keratins and visualised using anti-mouse HRP. There are significantly more colonies in $\beta1^{bri}/Dsg3^{dim}$ than $\beta1^{bri}/Dsg3^{bri}$ wells of both cell types. (C) Quantitative colony analyses include colony density (total colony area per dish) and CFE (ratio of colony number to plated cell number) (left graph), colony size (mean colony area) and colony number (mean colony number per dish) (right graph). There is a 4- or 5-fold increase in colony density, and a 7- or 3-fold increase in CFE in $\beta1^{bri}/Dsg3^{dim}$ compared with $\beta1^{bri}/Dsg3^{bri}$ in either thick or thin skin keratinocytes, respectively ($^*P<0.05$). Significantly more colonies are seen in $\beta1^{bri}/Dsg3^{dim}$ rather than $\beta1^{bri}/Dsg3^{bri}$ population ($^*P<0.05$) and larger colonies show in thin skin Kc ($^*P<0.05$).

Collectively, these data clearly demonstrate that labelling keratinocytes for β1 and Dsg3 together and sorting for a $\beta1^{bri}/Dsg3^{dim}$ population further enriches the putative epidermal stem cell compartment.

Discussion

Emerging evidence suggests that desmosomes not only function as cell adhesion structures involved in the maintenance of tissue integrity but also participate in the regulation of epithelial morphogenesis and cell positioning (Runswick et al., 2001), in signal transduction (Green and Gaudry, 2000) and in epithelial cell proliferation and differentiation as indicated by transgenic mouse studies (Allen et al., 1996; Merritt et al., 2002) and human genetic diseases (Armstrong et al., 1999; Norgett et al., 2000) (our unpublished data). These functions are accomplished by changes of desmosome composition and frequency across complex epithelia, a phenomenon consistently observed in skin, hair follicle, tongue, oesophagus and mucosa (Arnemann et al., 1993; Legan et al., 1994; North et al., 1996; Shirakata et al., 1998; Wan et al., 2003). In this study, we demonstrate for the first time that human adult palm basal keratinocytes exhibit a diversity of desmosome expression with a correlation between desmosome expression and different keratinocyte cell types in the basal layer of the skin (Fig. 1). We found that dissociated basal keratinocytes, fractionated on the basis of desmosome abundance, could produce different populations with differential properties of clonogenecity, CFE and long-term cell culture survival (Figs 3-6), suggesting that desmosomes are able to serve alone as a marker to enrich the putative epidermal/progenitor cells from adult skin. We find that the $Dsg3^{dim}$ cells are able to form significantly larger, expanding colonies, that result in higher colony density compared with $Dsg3^{bri}$ cells (Fig. 3), suggesting a significantly higher growth potential for the $Dsg3^{dim}$ cells. Moreover, the marked differential morphology and colony forming capacity between the two populations are most evident from 2 weeks (passage 2) onwards (Fig. 4 and data not shown). Only the cells in the $Dsg3^{dim}$ population exhibit exceptionally high proliferative potential and long-term survival capability, and those are salient characteristics of putative stem cells (Li et al., 1998). This approach is another example of prospective purification of human keratinocytes with high CFE and proliferative potential using a single cell surface adhesion molecule as was shown previously for the β1 integrin (Jones and Watt, 1993; Jones et al., 1995). However, in contrast to β1 integrin, Dsg3 serves as a negative marker for epidermal stem cell enrichment. Since desmosomes serve as anchors for the keratin cytoskeleton, this finding may support previous observation that putative non-serrated stem cells in monkey palm contain a paucity of keratin filaments (Lavker and Sun, 1983). It seems logical to reason that epidermal stem cells bear fewer desmosomes than non-stem cells. In support of this hypothesis are studies on other intercellular junctions which suggest that putative epidermal stem cells lack gap junctions and have low

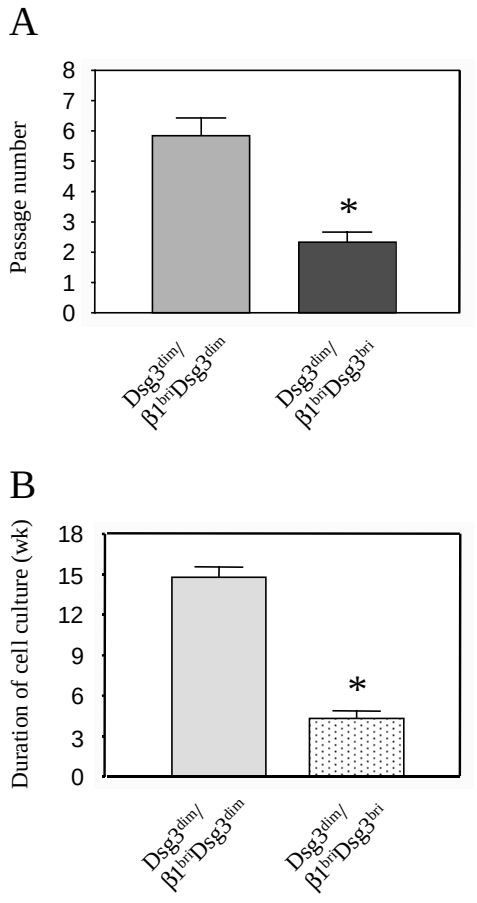


Fig. 6. Keratinocytes in Dsg3^{dim} orβ1^{bri}/Dsg3^{dim} populations have a greater overall proliferative potential than cells in the Dsg3^{bri} or β1^{bri}/Dsg3^{bri} populations. (A) Passage number and (B) duration of cell culture of different keratinocyte fractions in six independent experiments. The age of the skin donor was between 15 and 50 years old. Cells used for FACS were all from passage 1 following initial cultivation from the tiny biopsies. At each passage, cells were plated at the same density for each fraction. Note both the passage number and culture duration show significant differences (*P<0.05), and cells in Dsg3^{dim} or β1^{bri}/Dsg3^{dim} populations are capable of expanding more passages and surviving for a longer periods in culture than cells in the Dsg3^{bri} or β1^{bri}/Dsg3^{bri} populations.

expression of E-cadherin and β-catenin, the major constitutive proteins of zonula adherens (Matic et al., 1997; Moles and Watt, 1997; Akiyama et al., 2000; Matic et al., 2002). Another report provided evidence of only weak immunoreactivity for Dp, Pg and Pkp1 in the basal layer of outer root sheath of adult and foetal human scalp skin, where stem cells have been shown to reside (Kurzen et al., 1998). These findings suggest that the more primitive epidermal stem cells may possess few intercellular junctional complexes though they remain rich in cell-matrix adhesion molecules, such as integrins (Jones and Watt, 1993; Jones et al., 1995; Li et al., 1998), possibly reflecting the need for a stable adhesion to the extracellular matrix; and a weaker or non transient adhesion to neighbouring cells.

The distribution of stem cells, as identified by label-retaining or β1 integrin-bright cells, is not random but varies in location according to the specific phenotype of epithelial tissue (Jones and Watt, 1993; Jones et al., 1995). They may occur at the centre of the cluster of basal cells associated with each epidermal proliferative unit (Potten and Morris, 1988). They have been identified in the bulge of the outer root sheath in the hair follicle (Lenoir et al., 1988; Cotsarelis et al., 1990), in the limbus of the cornea (Cotsarelis et al., 1989), in the flat interpapillary basal layer of oesophageal epithelium (Seery, 2002), and at the tips of deep rete ridges in the palm (Lavker and Sun, 1983). These cells exist as clusters in tissue, rather than as sporadic single cells (Jones et al., 1995; Watt, 2002). In this study we demonstrate, by immunofluorescence and *in situ* hybridisation analysis, that there are clusters of cells at the tips of deep rete ridges in human adult palm having low levels of Dp and Dsg expression (Fig. 1A-C), suggesting a overlap of the desmosome-sparse cells and epidermal stem cells in this region. Studies on the existence of the desmosome-sparse cells on other stem cell locations in a variety of epithelial tissues are considered to be necessary.

We demonstrate that cell populations with high levels of β1 integrin (fractionated by rapid adhesion to type IV collagen (Jones and Watt, 1993), tend to have a low level of Dp and the other major desmosomal proteins (Fig. 2). Studies by others have suggested that the expression differences of cellular proteins between these two populations varied by up to only twofold (Watt, 2001). In this study we detected considerably greater fold differences in Dp expression although our flow cytometry data still indicated less than twofold differences in β1 integrin expression between these two populations (data not shown). Whether this greater expression of Dp was overestimated, possibly because of the presence of other cell types in culture requires further studies. Heterogeneous Dp expression in the rapidly adhering cells (β1-bright) may suggest the presence of some transit amplifying cells in this population, particularly using such a rapid adhesion assay, and also the progressive changes of cell type occurred with cell differentiation and maturity in this population (data not shown) (Jones et al., 1995; Li et al., 1998). It is therefore important to further identify and purify the truly stem cells within the β1-bright cell population.

The β1 integrins were initially proposed to be a useful cell surface marker for the enrichment of epidermal keratinocytes (Jones and Watt, 1993; Jones et al., 1995). More recently, questions about the purity of β1-bright stem cells have arisen, since these cells contain about 40% of the basal keratinocytes (Jones and Watt, 1993; Jones et al., 1995), far more than the 10% of basal cells predicted to be stem cells by cell kinetic studies (Potten and Morris, 1988; Jones et al., 1995; Li et al., 1998; Dunnwald et al., 2001; Birkenbach and Dunnwald, 2002). In this extended study based on β1 integrin (Jones and Watt, 1993; Jones et al., 1995), we demonstrate that a low level of the second cell surface marker, Dsg3, from the β1-bright cell population yields pronounced enrichment of cells with a high clonogenecity, CFE and, most importantly, a greater overall proliferative potential (Figs 5 and 6). This last feature is considered to be a very important characteristic for distinguishing the stem cells from transit amplifying cells, since both cell types are mitotic cells at early passages but only the stem cells are able to sustain in long-term generation of keratinocytes (Li et al., 1998). We find that using such stringent sorting criteria for the β1^{bri}/Dsg3^{dim} cells enables us to enrich

approximately 10% of the basal keratinocyte population. The culture duration of the $\beta1^{bri}$/$Dsg3^{dim}$ cells was ~14.3 weeks, in contrast to ~4.2 weeks in the $\beta1^{bri}$/$Dsg3^{bri}$ cells (Fig. 6). Our finding clearly indicates a subgroup in the β1-bright cell population, i.e. the $\beta1^{bri}Dsg3^{bri}$ cells that did not exhibit the stem cell characteristics and were unable to survive in long-term culture. As the $\beta1^{bri}$/$Dsg3^{bri}$ cells only exhibit a short-term survival (~4.2 weeks), it is speculated that they may represent the transit amplifying cells in the $\beta1^{bri}$ population. Collectively, these data clearly suggest that combining two markers together offers considerable advantages for the isolation of the putative keratinocyte stem cells, which provides another powerful tool to enrich the putative epidermal stem cells in addition to the combined markers of α_6 and $10G7^{dim}$ ($CD71^{dim}$) (Li et al., 1998; Tani et al., 2000). Surprisingly, both these approaches [our extended study based on β1 integrin and Li et al. (Li et al., 1998)] share some features in common. First, both studies use one of the integrin subunits (β1 or α_6) as the positive marker to enrich the stem and some transit amplifying cells with the high cell-matrix adhesive property as well as great growth capacity (Jones et al., 1995; Li et al., 1998). Second, both approaches apply the second negative marker, Dsg3 or differentiation-related CD71, to facilitate isolation of the putative stem cells from the transit amplifying cells, which yields approximately 10% of the basal keratinocyte population with properties of not only the high growth capacity but also the pronounced proliferative potential (high CFE and long-term duration of culture). Third, although different human skins and donor age (neonatal foreskin vs adult palm and mainly breast skins) are used in these studies, the duration of the cultures is similar [~12.7 weeks (Li et al., 1998) vs 14.3 weeks (in this study)]. Further characterisation of the stem cell properties of $Dsg3^{dim}$ and $\beta1^{bri}$/$Dsg3^{dim}$ cell populations, such as label-retaining and full skin regeneration capacities, is needed. Indeed, it would be of interest to further enhance a stem cell population combining two negative markers, connexin 43 (Matic et al., 1997; Matic et al., 2002) and $Dsg3^{dim}$ cells.

Unlike most other studies in the literature, where neonatal foreskin was the major source of the primary keratinocytes (Jones and Watt, 1993; Jones et al., 1995; Li et al., 1998), the keratinocytes generated in this study, particularly the palm cells, were from the small skin biopsies (~2-3 mm^2) of adults up to 50 years of age. Each strain varied according to body site, age and size of specimen. However, regardless of all these factors, we consistently obtained high clonogenecity, CFE and proliferative potential in the $Dsg3^{dim}$ or the $\beta1^{bri}$/$Dsg3^{dim}$ populations. Our findings provide a novel strategy for the improved purification of adult putative epidermal stem/progenitor cells that could pave the way for further studies in stem cell and desmosome biology, and also have implications for the development of new keratinocyte stem cell-mediated clinical treatments.

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