The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: a new view based on whole-mount labelling and lineage analysis

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SUMMARY

In order to examine the spatial organisation of stem cells and their progeny in human epidermis, we developed a method for whole-mount epidermal immunofluorescence labelling using high surface \( \beta_1 \) integrin expression as a stem cell marker. We confirmed that there are clusters of high \( \beta_1 \) integrin-expressing cells at the tips of the dermal papillae in epidermis from several body sites, whereas \( \alpha_6 \) integrin expression is more uniform. The majority of actively cycling cells detected by Ki67 or bromodeoxyuridine labelling were found in the \( \beta_1 \) integrin-dull, transit amplifying population and integrin-negative, keratin 10-positive cells left the basal layer exclusively from this compartment. When we examined p53-positive clones in sun-exposed epidermis, we found two types of clone that differed in size and position in a way that was consistent with the founder cell being a stem or transit amplifying cell. The patterning of the basal layer implies that transit amplifying cells migrate over the basement membrane away from the stem cell clusters. In support of this, isolated \( \beta_1 \) integrin-dull keratinocytes were more motile on type IV collagen than \( \beta_1 \) integrin-bright keratinocytes and EGFP-labelled stem cell clones in confluent cultured sheets were compact, whereas transit amplifying clones were dispersed. The combination of whole-mount labelling and lineage marking thus reveals features of epidermal organisation that were previously unrecognised.

Key words: Epidermis, Stem cell, Integrin, p53, GFP, Human, Lineage analysis

INTRODUCTION

The epidermis is one of the tissues in the human body that is maintained through proliferation of stem cells, cells that retain the ability to self-renew throughout adult life and also to produce daughter cells that undergo terminal differentiation to form the protective covering of the body (Lajtha, 1979; Hall and Watt, 1989; Morrison et al., 1997). Stem cells reside in the basal layer of the epidermis, attached to the underlying basement membrane. A further two subpopulations of basal keratinocytes have been defined by cell kinetic analysis and experiments on cultured keratinocytes (reviewed by Potten and Morris, 1988; Jones, 1997; Watt, 1998): transit amplifying cells and committed cells. Transit amplifying cells are stem cell daughters that undergo a small number of rounds of division within the basal layer, estimated at 3-5 (Potten, 1981; Jones and Watt, 1993; Jones et al., 1995). Committed cells are transit amplifying cell daughters that are destined to move upwards from the basal layer by a process that involves downregulation of integrin expression and function (Adams and Watt, 1990; Hotchin et al., 1995), thereby withdrawing from the cell cycle and undergoing terminal differentiation.

For many years, studies of epidermal stem cells were hampered by the absence of any molecular markers that could be used to enrich for stem and transit amplifying cells, the type of marker that has been used to great effect in studies of haemopoietic stem cells (Heyworth et al., 1997). However, we have found that high surface \( \beta_1 \) integrin expression is a marker of stem cells, both in vivo and in cultures of primary human keratinocytes (Jones and Watt, 1993; Jones et al., 1995; Gandarillas and Watt, 1997; Zhu and Watt, 1999), the stem cell population expressing 2- to 3-fold more surface integrins, as determined by flow cytometry, than the transit amplifying cells. While not all \( \beta_1 \) integrin-bright cells are stem cells (about 40% of cells in the basal layer are integrin-bright, but the percentage of stem cells in the basal layer is closer to 10%; Jones et al., 1995), integrin levels can nevertheless be used to reveal the distribution of stem cells in the epidermis (Jones et al., 1995; Molèes and Watt, 1997).

Integrin-bright and -dull keratinocytes are found in patches, or clusters, that have a specific location with respect to the epidermal-dermal junction (Jones et al., 1995). In interfollicular epidermis of foreskin, breast and scalp, the integrin-bright patches lie at the tips of the dermal papillae, where the dermis comes closest to the skin surface (see Fig. 1), whereas, in the thickened epidermis characteristic of palm...
and sole, the integrin-bright cells lie at the tips of the deep rete ridges, where the epidermis projects furthest into the skin (Jones et al., 1995; see also Lavker and Sun, 1983). When the epidermis is in a steady state, the stem cells are believed to be infrequently in S phase of the cell cycle (slow cycling cells; Potten and Morris, 1988; Cotsarelis et al., 1990) and, consistent with this view, there is a higher proportion of S-phase cells in integrin-dull compared with integrin-bright patches of the epidermal basal layer (Jones et al., 1995). The patterning of stem and transit amplifying cells can be recreated in culture and is subject to autoregulation (Jones et al., 1995).

In order to improve our understanding of how cells in the epidermal basal layer are arranged, two developments are necessary. The first is to be able to visualise integrin-bright and -dull regions in whole mounts of intact epidermis, as it is difficult to obtain an accurate impression of three-dimensional tissue organisation when relying solely on two-dimensional tissue sections (Mehregan and Hashimoto, 1991; Iizuka et al., 1996, 1997). The second is to be able to apply lineage marking to the system in order to determine the spatial relationship of individual integrin-bright stem cells to their integrin-dull and integrin-negative (i.e. suprabasal) progeny. In this report, we describe our progress towards achieving these objectives.

MATERIALS AND METHODS

Preparation of whole mounts

Normal human skin was obtained from neonatal circumcisions (foreskin) or adult plastic surgery operations (breast, eyelid). The fat and most of the connective tissue was removed using curved scissors. The skin was divided into 1-1.5 cm² pieces and incubated in 50 mg/ml Dispase II (Boehinger Mannheim) in DMEM buffered with 20 mM Hepes on ice for 18-22 hours. Using forceps, the epidermis was gently removed from the underlying dermis as an intact sheet and fixed immediately in normal buffered formalin, pH 7.2, (Sigma) for 2 hours at room temperature. In some experiments, epidermal sheets were incubated in complete keratinocyte medium (FAD+FCS+HICE; Jones et al., 1995) supplemented with 65 µM bromodeoxyuridine (BrdU) in 20 mM Hepes, pH 7.2, at 37°C for 1 hour prior to fixation. Fixed epidermal sheets were stored in PBS containing 0.2% sodium azide at 4°C for up to 8 weeks prior to staining.

Antibodies

For confocal microscopy, β1 integrins were detected using mouse monoclonal antibody P5D2 (Dittel et al., 1993; Developmental Studies Hybridoma Bank), unconjugated or conjugated to Alexa 488 or 594 (Molecular Probes Inc.), or rat monoclonal antibody mAb 13 (Akiyama et al., 1989; Becton Dickinson), unconjugated. Alexa protein labelling kits (Molecular Probes, Inc) were used to conjugate the Alexa dyes to P5D2. For FACS analysis, FITC-labelled anti-CD29 (Dittel et al., 1993; Developmental Studies Hybridoma Bank), unconjugated or conjugated to Alexa 488 or 594 (Molecular Probes Inc.), or rat monoclonal antibody mAb 13 were detected with secondary antibodies conjugated to Alexa dyes purchased from Molecular Probes Inc. The Alexa fluorocromes 488 and 594 were chosen because they are very photostable and thus there was minimal photobleaching during capture of confocal images. Alexa 488 excitation/emission spectra are in the same range as FITC and those of Alexa 594 are equivalent to Texas Red.

Immunostaining

Epidermal sheets were simultaneously blocked and permeabilised by incubation in 0.5% skim milk powder, 0.25% fish skin gelatin (Sigma), 0.5% Triton X-100 in TBS (0.9% NaCl, 20 mM Hepes, pH 7.2) (PB buffer) for 30 minutes. The primary antibodies were diluted in PB buffer and incubated at room temperature overnight with gentle agitation. The epidermis was then washed in PBS containing 0.2% Tween 20 for 3-4 hours with several changes of wash buffer. The incubation with secondary antibodies was performed in the same way. The labelling procedure for detecting p53 was that described by Jonason et al. (1996) with the following minor modifications: epidermal sheets were incubated for 20 minutes in 3% H₂O₂ in methanol to reduce background and Cy3 Renaissance was used for the visualisation. After permeabilisation and prior to incubation with anti-BrdU antibody, BrdU-labelled sheets were treated for 15 minutes at 37°C with 50 U/ml DNAase (Sigma DN-25) dissolved in PBSABC (PBS supplemented with 1 mM CaCl₂ and 1 mM MgCl₂) and made up immediately before use (Carayon and Bord, 1992).

When sheets were double labelled with two primary mouse monoclonal antibodies, they were incubated in the following sequence: first primary antibody, secondary antibody, second primary antibody directly conjugated with fluorochrome. A blocking step using 1% mouse serum in PBS was introduced before the addition of
the second primary mouse antibody. 1% mouse serum was also added during incubation with the second primary antibody.

Cryosections of whole mounts stained with PSD2 and visualised using Alexa 488 were collected onto Frost Plus slides and dried for 2 hours at 37°C. The sections were blocked in TBS containing 0.5% skim milk powder and 0.25% fish skin gelatin (Sigma) without Triton X-100 for 30 minutes and incubated with mAb 13 followed by Alexa 594-conjugated goat anti-rat antibody.

Confocal microscopy
A Zeiss 510 confocal microscope was used. 50-100 optical sections of each epidermal sheet were captured with an increment of 0.5-1.0 μm. Objectives used were Zeiss 10/NA 0.45, Zeiss 20/NA 0.75 and Zeiss 63/NA 1.4. The line average was set to 8. Scans are presented as z-projections or calculated vertical sections from the scan stack. The samples were scanned from the dermal side towards the epidermal surface to a total thickness of 40-60 μm, which encompassed the entire basal layer overlying rete ridges and dermal papillae.

Quantification was performed as follows. Using the Lookup Tables (LUTs) provided as part of the computer software, false colour was applied to the images by designating the pixel intensities (range: 0-255 units) different codes: blue for the top 1/3 (i.e. brightest fluorescence), red for the bottom 1/3 (i.e. dullest) and green for the remaining 1/3 (i.e. medium) pixel intensities. In order to correlate our data with the earlier results of Jones et al. (1995), the brightness histograms from the β1 integrin staining were shifted so that 30% of the sample area (i.e. 40% of the basal layer after application of area correction factor 1.33, described in Results) fell within the top 1/3 of fluorescence intensity (blue area). This false colour image was analysed using IPLab (Signal Analytics Inc., USA) with the segmentation tool. The β1 integrin-bright segments defined in this way included the majority of the epidermis over the dermal papillae. The resulting segments were added to the channel in which K67 antigen or BrdU was detected and the positive nuclei were scored both inside and outside the segments.

Cell motility
Normal human keratinocytes derived from neonatal foreskin (km and kq, passages 2-7) were grown on 3T3 feeders as described previously (Rheinwald, 1989; Jones and Watt, 1993). The culture medium (FAD+FCS+HICE) consisted of one part Ham’s F12 medium and three parts DMEM supplemented with 1.8×10^4 M adenine, 10% foetal calf serum, 0.5 μg/ml hydrocortisone, 5 μg/ml insulin, 10^-10 M cholera toxin and 10 ng/ml epidermal growth factor (Jones and Watt, 1993). At subconfluence, the cells were harvested by trypsinisation, washed and stained on ice for 20 minutes with FITC-conjugated anti-cd29 diluted in FAD+FCS+HICE. The cells were washed and propidium iodide was added in order to gate out dead cells (Jones and Watt, 1993). Live basal cells were sorted into β1 integrin-bright and -dull populations as described previously (Jones and Watt, 1993), except that the 10-15% brightest and dullest cells were isolated rather than the brightest and dullest 20%. The sorted cells were plated in DMEM supplemented with 10 ng/ml EGF on bacteriological plastic dishes (Falcon) that had been coated with 50 μg/ml human collagen type IV (Sigma) in PBSABC at 4°C overnight and blocked in heat-inactivated 0.5% BSA at 37°C for 30 minutes. The cells were kept humidified at 37°C with 5% CO2 and videotaped for 20-22 hours with 5 minutes between frames. Motility was measured from digitised films using a cell tracking extension (ICRF) written for IPLab (Signal Analytics Inc., USA).

EGFP-labelled keratinocytes
An enhanced variant of green fluorescent protein with a red-shifted excitation spectrum (EGFP; Clontech) was subcloned into the BamHI-Sall sites of the retroviral vector pBabe puro (Morgenstern and Land, 1990). 20 μg of retroviral DNA was transfected into the ecotropic packaging cell line GP+E (Markowitz et al., 1988) using Superfect (Qiagen) according to the Manufacturer’s instructions. After 2 days, 2.5 μg/ml puromycin was added to select for transduced cells. After 1 week, cells with high EGFP expression (brightest 30%) were selected by FACS and allowed to grow to confluence. Cells were then rinsed and incubated in puromycin-free medium for 48 hours to collect ectopic retroviruses. The virus-containing medium was harvested, centrifuged to remove cells, supplemented with 8 μg/ml polybrene and incubated with 2×10^5 amphotropic GP+env AM12 packaging cells (Markowitz et al., 1988) for 16 hours. The AM12 cells were selected with puromycin and then by FACS in the same way as described above for GP+E cells. Retroviral packaging cell lines were grown in DMEM containing 10% FCS.

Normal human epidermal keratinocytes from neonatal foreskin (strain kq, passages 2-4) were transduced with EGFP by coculture with a polyclonal population of AM12 producer cells that had been pretreated with 4 μg/ml mitomycin C (Sigma). Transduced cells were selected in 2.5 μg/ml puromycin as described previously (Gandarillas and Watt, 1997).

Populations enriched with stem cells and transit amplifying cells were selected on the basis that stem cells adhere more rapidly to type IV collagen (Jones and Watt, 1993). Briefly, tissue culture plastic dishes (Falcon) were coated overnight at 4°C with 10 μg/ml type IV collagen (Sigma) and blocked with 0.5 mg/ml heat-denatured BSA (Sigma) at 37°C for 1 hour. EGFP-expressing keratinocytes (5×10^5/cm^2) were allowed to attach to the dishes for 20 minutes at 37°C to enrich for stem cells. Nonadherent cells were removed and plated on a separate collagen-coated dish; those that attached within 60 minutes constituted the transit amplifying population. Note that this transit cell enrichment method differs from that of Jones and Watt (1993) and the degree of enrichment is reduced as a result. After this procedure, each dish contained 150-200 EGFP-expressing keratinocytes/cm^2 as determined by direct counting. 10^5/cm^2 uninfected keratinocytes were added to each dish of EGFP-transduced keratinocytes to achieve a confluent layer of cells within 24 hours.

Cells were either fixed on the dish in 4% formaldehyde 5 days after plating, or removed as a sheet using 2.5 mg/ml Dispase 7 days after plating. EGFP-positive colonies of keratinocytes were scored using a Zeiss Axioskop fluorescence microscope (Carl Zeiss Ltd, Herts, UK). Dispase sheets of keratinocytes were stained for β1 integrins using Alexa 594-conjugated PSD2 and the distribution of EGFP-positive clones relative to integrin-bright patches was analysed on a Zeiss 510 upright confocal microscope.

RESULTS

Development of whole-mount labelling method
Three methods for separating the epidermis from the underlying dermis were evaluated: heat treatment (56°C for 3 minutes), incubation with thermolysin (Jones et al., 1995) or incubation with Dispase (as described in Materials and Methods). Epidermal sheets obtained by each method were labelled with a mouse monoclonal antibody to β1 integrins (PSD2-conjugated to Alexa 488) and then frozen sections were incubated with the second primary mouse antibody. 1% mouse serum was also added during incubation with the second primary antibody.

PERMEABILISATION USING ORGANIC SOLVENTS (METHANOL, ETHANOL OR ACETONE) LEAD TO LOSS OF β1 INTEGRIN STAINING, WHEREAS STAINING WAS PRESERVED FOLLOWING PERMEABILISATION WITH TRITON X-100.
With optimal permeabilisation conditions, whole mounts prepared with Dispase gave complete colocalisation of P5D2 and mAb 13 labelling and a stronger P5D2 signal than that obtained in whole mounts prepared using heat or thermolysin treatment (data not shown). The reason was that heat- and thermolysin-detached sheets had residual basement membrane attached, as verified by immunostaining with an antibody to type IV collagen, whereas Dispase sheets did not (data not shown).

Having optimised the whole-mount labelling conditions, we restricted our analysis to foreskin, breast and eyelid skin, body sites in which the β1 integrin-bright patches overlay the dermal papillae (Fig. 1; Jones et al., 1995, and the present report). Fig. 2A shows the image obtained from whole-mount labelling of foreskin epidermis with an anti-β1 integrin antibody. The basal layer is viewed from the perspective of the dermis. In Fig. 2C a false colour scale has been applied to the image shown in Fig. 2A: the β1 integrin-bright cells, visualised in blue on the scale, are at the tips of the dermal papillae, separated from one another by areas of integrin-dull cells, visualised in red. This is further illustrated in Fig. 2F, in which a vertical section was calculated from the z stack through the field at the position of the yellow line in Fig. 2C. The vertical section shows how the basal layer undulates, with β1 integrin-bright cells clustered at the tips of the dermal papillae. Within the bright patches, cells often appeared to radiate from a single central cell (Fig. 2A, insert).

Both the size of the bright patches and proportion of integrin-bright basal cells were consistent with the observations of Jones et al. (1995) on vertical sections of foreskin epidermis. Thus the bright patches were approximately 14 cells in diameter, as reported previously (Jones et al., 1995) and the proportion of integrin-bright basal cells was about 30% of the flat confocal projections. Based on the observation that the height of a dermal papilla is about half its diameter (in the body sites examined) and assuming that its tip has the surface area of a quarter of a sphere (sphere cap), the true proportion of integrin-bright cells is 30% × 1.33 = 40%, close to the percentage of integrin-bright cells in foreskin reported by Jones et al. (1995) (43.0±1.7%). The multiplication factor was calculated from the formula A_cap/A_proj = 1/1 − h/D, where A_cap is the area of the sphere cap, A_proj is the area of the flat projected surface (i.e. the integrin-bright patch in the flat confocal image), h is the height of the cap and D is the diameter of the sphere.

We have previously shown that α6 integrin levels show little variation between β1 integrin-bright and -dull keratinocytes (Jones et al., 1995) and as a further validation of the whole-mount methodology, we double labelled epidermal sheets for β1 and α6 integrin subunits, as illustrated in Fig. 2A,B. The α6 staining was concentrated at the centre of the ventral plasma membrane of individual cells, underneath the nucleus (Fig. 2B), whereas the β1 staining was concentrated at cell-cell borders (Fig. 2A). At higher power, the relative distribution of the two integrins

**Fig. 2.** Confocal micrographs of a whole mount of foreskin epidermis stained for α6 and β1 integrins by double-label immunofluorescence. (A) β1 integrins visualised using Alexa 488. In some integrin-bright patches, there is a central cell from which the others appear to radiate: this is illustrated (insert) in the case of one cluster (arrow) by showing a single section from the z stack. In the inset the magnification has been increased 1.5-fold and the contrast enhanced. (B) α6 integrins visualised using Alexa 594. (C,D) False colour images of A and B, respectively. The strongest fluorescence signal is translated into blue and the weakest into red. The yellow line in C,D shows the region of the whole mount used to calculate the vertical sections shown in E,G, respectively, in which the undulation of the basal layer is clearly seen. (E) At high power, the relative distribution of β1 (green) and α6 (red) integrins on the plasma membrane in contact with the basement membrane can be seen. Scale bars: 100 μm (A-D,F,G); 20 μm (E).
could be seen more clearly (Fig. 2E): punctate α6 staining, corresponding to individual hemidesmosomes (Stepp et al., 1990; Sonnenberg et al., 1991) was concentrated in the centre of the ventral plasma membrane of basal cells; there was some diffuse β1 integrin staining interspersed with the α6 staining, but the majority of the β1 integrin was concentrated at the cell periphery, forming a ring at the base of each cell.

Quantitation of the α6 integrin fluorescence by application of the false colour scale is shown in Fig. 2D. Whereas the β1 integrin-bright cells were clustered at the tips of the dermal papillae (Fig. 2C), there was no such correlation for α6. This is also illustrated in the vertical section shown in Fig. 2G where the distribution of the α6 integrin-bright cells is more uniform than that of the β1 integrin-bright cells (Fig. 2F).

**Position of proliferating and differentiating basal cells relative to the stem and transit amplifying compartments**

Comparison of the proportion of actively proliferating keratinocytes in different regions of the basal layer by examination of histological sections is extremely laborious, as many individual sections must be scored to obtain statistically significant data (Jones et al., 1995; reviewed by Potten and Morris, 1988). We therefore applied the whole-mount labelling technique to the problem. We used two methods to visualise proliferating cells in epidermal sheets from breast (Fig. 3; Table 1) and foreskin (data not shown) immunolabelled for β1 integrins. The first was to stain the sheets with an antibody to Ki67, a nuclear protein expressed by proliferating cells (Schlüter et al., 1993) (Fig. 3A) and the second was to incubate freshly isolated sheets for 1 hour with BrdU to label S-phase cells (Fig. 3B). The intensity of β1 integrin labelling was
Table 1. Whole mounts of breast skin stained for β1 integrins and Ki67 or BrdU. The confocal projections were translated into colour code based on the β1 integrin signal.

<table>
<thead>
<tr>
<th></th>
<th>Integron-dull area %</th>
<th>Positive nuclei</th>
<th>Frequency of positive nuclei</th>
<th>Integron-bright area %</th>
<th>Positive nuclei</th>
<th>Frequency of positive nuclei</th>
<th>Frequency ratio</th>
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<tbody>
<tr>
<td>Ki67</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field 1</td>
<td>68.44</td>
<td>218</td>
<td>3.19</td>
<td>31.56</td>
<td>20</td>
<td>0.63</td>
<td>5.03</td>
</tr>
<tr>
<td>Field 2</td>
<td>80.50</td>
<td>203</td>
<td>2.50</td>
<td>19.50</td>
<td>7</td>
<td>0.36</td>
<td>6.90</td>
</tr>
<tr>
<td>Field 3</td>
<td>76.00</td>
<td>182</td>
<td>2.39</td>
<td>24.00</td>
<td>14</td>
<td>0.58</td>
<td>4.12</td>
</tr>
<tr>
<td>BrdU</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field 1</td>
<td>74.60</td>
<td>66</td>
<td>0.88</td>
<td>25.40</td>
<td>11</td>
<td>0.43</td>
<td>2.05</td>
</tr>
<tr>
<td>Field 2</td>
<td>70.84</td>
<td>120</td>
<td>1.69</td>
<td>29.16</td>
<td>15</td>
<td>0.51</td>
<td>3.31</td>
</tr>
<tr>
<td>Field 3</td>
<td>71.14</td>
<td>98</td>
<td>1.37</td>
<td>28.76</td>
<td>9</td>
<td>0.33</td>
<td>4.41</td>
</tr>
</tbody>
</table>

The integrin-bright areas were segmented using IPLab and the percentage of the total area of the basal layer occupied by integrin-bright or -dull cells was determined. Table 1 shows data from three microscopic fields (from three different whole mounts prepared from two different donors) for each marker. In each case, the observed frequency of proliferating cells was significantly higher in the integrin-bright areas than the integrin-dull areas. 

A high-power view of a β1 integrin-dull region is shown in Fig. 4B-E: each of the three cells that has been marked with arrows is attached to the basement membrane zone via a thin stalk (Fig. 4B) while the nucleus and most of the cell body is suprabasal, overlying neighbouring β1 integrin-expressing cells (Fig. 4F). The shape of two of the keratin 10-positive cells is revealed in the vertical section in Fig. 4E, calculated from a z stack at the position of the yellow line in Fig. 4D.

**p53 as a lineage marker**

A prediction of our model is that if the progeny of individual epidermal cells could be identified, two types of clone should be found and the boundaries between clones should lie in the transit amplifying compartment. Jonason et al. (1996) and Ren et al. (1997) have reported the existence in sun-exposed human skin of benign clonal patches of keratinocytes with p53 mutations that show no evidence of malignant transformation. p53 mutations stabilise the protein, resulting in nuclear positivity (Greenblatt et al., 1994). We examined the size and distribution of these patches with respect to the stem and transit amplifying compartments. Clinically normal epidermis from the eyelids of patients undergoing plastic surgery was subjected to whole-mount labelling with an antibody to p53, essentially as described by Jonason et al. (1996). Out of a total of 12 eyelids, 9 contained p53 patches.

Whole mounts labelled for p53 had to be treated with methanol and H$_2$O$_2$ to reduce the background staining and this destroyed the integrin epitopes. However, the distribution of Ki67-positive and integrin-bright cells with respect to the dermal papillae was the same in eyelid as in breast and foreskin (data not shown) and therefore we analysed those p53 clones that lay in areas with distinct dermal papillae. In this way, only 6 out of a total of 43 patches proved informative as the dermoeipidermal junction is flat in most of the eyelid (Brieggaman et al., 1991). In four cases, the p53 clone included several stem cell clusters (Fig. 5A) while, in two cases, the patch was confined to the transit amplifying compartment and excluded from the neighbouring dermal papillae (Fig. 5B). In every case, the boundaries between the p53-positive clone and neighbouring p53-negative cells fell in the transit amplifying compartment.

**Relative motility of stem and transit amplifying cells**

Since the β1 integrin-bright cells are clustered and give rise to...
all the other cells in the basal layer (Jones and Watt, 1993; Jones et al., 1995), there must be movement of transit amplifying cells away from the stem cell clusters. To test the prediction that transit amplifying cells are more motile than stem cells, β1 integrin-bright and -dull basal keratinocytes were selected from primary cultures by FACS, essentially as described previously (Jones and Watt, 1993), plated on type IV collagen and monitored by time-lapse videomicroscopy for 20-22 hours. As shown in Fig. 6A, the mean motility of the β1 integrin-dull cells was significantly greater than that of the integrin-bright cells (P<0.01, Mann-Whitney test). The range of average speeds was 0.27-0.43 μm/minute for the integrin-bright cells (6 experiments) and 0.41-0.65 μm/minute for the integrin-dull cells (7 experiments). 45-50 cells were scored per experiment.

To test whether the higher motility of the transit amplifying cells correlated with a more scattered distribution in an intact epidermal sheet, we used enhanced green fluorescent protein (EGFP) as a lineage marker in cultured keratinocytes. 100% of the target population was transduced with a retroviral vector expressing EGFP with puromycin as a selectable marker, using methods that have been described previously (Zhu and Watt, 1996; Gandarillas and Watt, 1997). EGFP-labelled stem and transit amplifying cells were selected from primary cultures by FACS, essentially as described previously (Jones and Watt, 1993; Jones et al., 1995), there must be movement of transit cell founders from which they are derived (Fig. 1, upper panel), a property that is not immediately apparent (Fig. 1). The first is that transit amplifying cells must move away from the stem cell clusters from which they are derived (Fig. 1, upper panel), a property that is not required in models in which each individual stem cell is surrounded by its transit amplifying daughters in the basal layer

combined with β1 integrin labelling in sheets that had been detached from the culture dish using Dispase. Clones were detected that either lay exclusively within an integrin-dull region (data not shown) or encompassed both integrin-bright and -dull areas (Fig. 6D,E), the former clones being characteristic of transit cell founders and the latter of stem cell founders. 20 stem and 20 transit amplifying cell colonies were examined. EGFP-positive cells in the integrin-dull areas were scattered while those in the integrin-bright areas were compact; both compact and scattered cells could be found within a single stem cell clone (Fig. 6D,E). Large, suprabasal, integrin-negative cells were also found in the clones and they sometimes overlay integrin-bright areas that did not contain any EGFP-positive cells (Fig. 6D,E).

In the clone illustrated in Fig. 6D,E, a false colour scale has been applied to the integrin fluorescence (Fig. 6E) in the same way as in Fig. 2C. The EGFP-positive cells in the area with the highest level of β1 integrins (labelled s) are compact whereas the cells within the adjacent region (labelled ta) are scattered. Suprabasal, terminally differentiating, cells are also shown (labelled td).

**DISCUSSION**

We have demonstrated that whole-mount labelling of sheets of normal human epidermis can be used to visualise the β1 integrin-bright and -dull areas of the basal layer that are enriched for stem and transit amplifying cells, respectively. The number and size of the β1 integrin-bright areas observed in the whole mounts were in good agreement with those determined from vertical histological sections (Jones et al., 1995). We confirmed that cells expressing high levels of the α6β4 integrin were not clustered and were uniformly distributed between the β1 integrin-bright and -dull regions; we also confirmed that actively cycling cells were largely confined to the β1 integrin-dull compartment (see Jones and Watt, 1993; Jones et al., 1995 and references cited therein).

When viewed in three dimensions, two new aspects of the relationship between stem cells and their progeny are immediately apparent (Fig. 1). The first is that transit amplifying cells must move away from the stem cell clusters from which they are derived (Fig. 1, upper panel), a property that is not required in models in which each individual stem cell is surrounded by its transit amplifying daughters in the basal layer...
The second is that the transit amplifying compartment is essentially continuous throughout the basal layer and in any given region will be derived from multiple adjacent stem cell clusters (Fig. 1, bottom panel). This latter feature is strikingly similar to the situation in the small intestine, where the differentiating cells on any given villus are derived from the stem cells of several adjacent crypts (Schmidt et al., 1988).

Cell motility depends on an intermediate level of integrin expression and at lower or higher levels motility is inhibited (Huttenlocher et al., 1995). Thus, a priori, the β1 integrin-dull transit amplifying cells should be more motile than the integrin-bright stem cell population. We now have experimental support for this: the motility of isolated transit amplifying cells on type IV collagen was greater than that of stem cells; and in confluent cultured sheets of keratinocytes in the transit amplifying clones were more dispersed than in the stem clones.

The compact phenotype of EGFP-marked stem cell clones and the dispersed phenotype of transit cell clones in confluent sheets of cultured keratinocytes could be due solely to differences in motility, but they may also reflect differences in intercellular adhesiveness. Indeed cell-cell and cell-extracellular matrix adhesiveness are not totally independent properties in keratinocytes (Hodivala and Watt, 1994) and other cell types (Huttenlocher et al., 1998). If intercellular adhesiveness does vary within the basal layer, stem cells will tend to be maintained in clusters through a process of cell sorting if they are more cohesive than their transit amplifying daughters (Mölès and Watt, 1997; Godt and Tepass, 1998), while transit cells derived from neighbouring stem cell clusters will be able to intermingle (as seen in Fig. 6C-E) because they are as cohesive as one another. Indirect support for this idea comes from the observation that the p53-positive clones attributed to transit cell founders were excluded from the surrounding stem cell clusters (dermal papillae; Fig. 5B).

A remarkable finding from whole-mount labelling was that all of the keratin 10-positive basal cells lay within the transit amplifying compartment and thus that departure from the basal layer did not occur randomly along the basement membrane. The issue of whether or not entry into the suprabasal compartment is random was considered by earlier workers. Iversen et al. (1968) concluded that in mouse epidermis upward migration is not random, the suprabasal transition being made by the oldest of the G1 neighbours adjacent to a dividing cell. Human keratinocytes can initiate terminal differentiation at any phase of the cell cycle (Dover and Watt, 1987; Gandarillas and Watt, 1997) and our observation that both S-phase cells and keratin 10-positive cells were concentrated within the same region of the basal layer is in good agreement with the finding of Régnier et al. (1986) that basal cells expressing keratins 1 and 10 are more likely to be in S/G2/M of the cell cycle than other basal cells. The fact that epidermal sheets can be BrdU labelled offers the potential to examine the relationship between the cell cycle and transition to the suprabasal layers in more detail.

Our finding that access to the suprabasal layers is solely via the transit compartment implies some degree of lateral cell movement not only within the basal layer but also within the suprabasal layers. There is preliminary evidence for this from the experiments with EGFP-labelled keratinocytes in culture, since labelled suprabasal cells were sometimes found overlying unlabelled basal cells (Fig. 6D,E). In intact epidermis, Jonason et al. (1996) found conical-shaped p53-positive clones with the apex in the basal layer, again demonstrating that suprabasal cells may overlie basal cells to which they have no lineage relationship. Nevertheless, since cell size increases during terminal differentiation (e.g. Watt and Green, 1981) and there are fewer suprabasal layers above the dermal papillae than the rete ridges, suprabasal cells will still tend to lie over their stem cell mother patch. This issue can best be explored further by applying lineage marking to xenografts of cultured human keratinocytes on immunocompromised mice, since the resulting
epidermis can be maintained for longer and has better morphology than cultured epidermis on tissue culture plastic (Klodkda et al., 1998; see also Ng et al., 1997; Iizuka and Ishida-Yamamoto, 1997).

Lineage marking of normal keratinocytes in situ in human epidermis is not feasible. However, analysis of clones of keratinocytes in which p53 had been stabilised through mutation did provide some information about lineage relationships. p53 mutations are believed to confer resistance to UV-induced apoptosis and, after surviving irradiation, the mutant cells clonally expand into areas vacated by those keratinocytes that have been killed by UV light (Ziegler et al., 1994; Jonason et al., 1996). The p53 patches have very little precancerous potential and thus the only result of mutation is benign clonal expansion (Jonason et al., 1996; Ren et al., 1997). Our prediction was that the p53 clones would be of two types, depending on whether the target cell in which p53 was mutated was a transit amplifying cell or a stem cell. The transit amplifying cells would be excluded from the stem cell compartment, i.e. overlying the dermal papillae, as their progeny would also be transit amplifying cells, albeit with increased proliferative potential, while the targeted stem cell clones would colonise both compartments. If our interpretation is correct, the p53-positive transit amplifying clones should disappear through terminal differentiation and thus have no likelihood of developing into tumours. It was interesting to find p53 patches encompassing multiple dermal papillae since in culture individual stem cells can also give rise to multiple stem cell (i.e. integrin-bright) patches (Jones et al., 1995). It is disappointing that we did not have a larger number of informative clones, but this was due to the small number of clones in the skin that we examined and also to the fact that the dermal papillae are absent in large areas of eyelid skin (Briggaman et al., 1991). The frequency of p53 clones was much lower than previously reported (Jonason et al., 1996; Ren et al., 1997) and may reflect the prevailing weather conditions in Northern Europe over the past two summers.

In conclusion, the application of whole-mount labelling to human epidermis has provided new insights into the organisation of stem cells and their progeny and offers considerable opportunities for further study. The ease with which the integrin-bright patches can be visualised allows us to evaluate other potential stem cell markers and to compare cell cycle parameters in the different keratinocyte subpopulations. It will also be possible to address the question of whether all cells in an integrin-bright patch are equivalent or whether there is a hierarchy with respect to stem cell characteristics. In cultured epidermis lineage marking with EGFP can be extended to explore lateral movement in the basal and suprabasal layers in more detail and to examine the factors that control stem cell number and density.

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