

INVESTIGATIVE REPORT

Functional Characterization of β_1 -Integrin-positive Epidermal Cell Populations

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Epidermal keratinocytes are heterogeneous and can be divided into stem cells (strong β_1 -integrin expression) with unlimited clonogenic potential, transient amplifying cells (weaker β_1 -integrin expression) with restricted proliferative capacity and terminally differentiated cells (no β_1 -integrin expression) that have lost the capacity to divide. We tested the hypothesis that cell kinetic characteristics of the epidermal subpopulations differ. Single cell suspensions from small human skin punch biopsies were sorted flow cytometrically into a β_1 -integrin weakly positive (dim) and strongly positive (bright) subpopulation and the clonogenic potential was compared in cell culture experiments. Image analysis was used to determine growth characteristics of the colonies. We found that cell size in the β_1 -integrin bright subpopulation increased when colonies aged, whereas this was constant in the dim subpopulation. The total number of colonies formed and the growth rate of the colonies were higher in the β_1 -integrin dim cells than in the bright subpopulation. Experimental data from this study confirm the hypothesis that cell kinetic characteristics of β_1 -integrin dim and bright cells are different. Combining flow cytometric sorting, cell culture and image analysis provides powerful means for phenotypical and functional characterization of epidermal subpopulations. **Key words:** stem cells; keratinocytes; flow cytometry; cell culture; image analysis.

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The epidermis forms the outer part of skin and is thought to contain putative epidermal stem cells (ESC). These cells are located in the basal layer, which is adjacent to the dermis. β_1 -Integrin, a cell surface glycoprotein, can be used to distinguish basal (strong β_1 -integrin expression) keratinocytes from suprabasal cells (weak or no β_1 -integrin expression) (1). β_1 -Integrin is known to play a role in keratinocyte differentiation and tissue assembly (2). The putative ESC form a subpopulation of cells that strongly express β_1 -integrin

(β_1 -integrin bright cells) (3–5) and have a high level of adhesiveness to extracellular matrix ligands. The ESC have an estimated cell cycle time of 100–300 h (6). They divide asymmetrically into a new ESC and a cell committed to progressive differentiation, which is called a transient amplifying cell (TAC). TAC only weakly express β_1 -integrin (β_1 -integrin dim cells) (3, 7). On account of their limited proliferative potential (4, 8–11), it is considered that they can undergo maximally five rounds of cell division and have a shorter cell cycle time (35–40 h) (12). The third subpopulation of keratinocytes consists of suprabasally located terminally differentiated cells that do not express β_1 -integrin and have lost the ability to divide.

Keratinocytes form a phenotypically and functionally heterogeneous population. In cell culture, three types of colonies are formed. The first type are holoclones, which are characterized by a high proliferative potential without terminal differentiation. The second type are the paraclones, which have a limited dividing capacity and undergo terminal differentiation afterwards. The third group is formed by the meroclones that are supposed to form a transitional stage between holoclones and paraclones (13, 14). Furthermore, cell adhesion assays showed that keratinocytes that adhered most rapidly to type IV collagen and fibronectin have a higher colony-forming efficiency than slowly adhering cells (3, 5, 15, 16). On the basis of these morphologic and qualitative differences, it was hypothesized that epidermal subpopulations also differ with respect to cell kinetic characteristics. Previous studies already showed that the growth potential of β_1 -integrin bright cells exceeds that of the dim cells (3, 4, 17). Our goal in this study was to characterize epidermal subpopulations on the basis of phenotype and to define different epidermal subpopulations with regard to cell kinetic features. Therefore, we made use of small human skin punch biopsies, which allow the use of human clinical material without considerable discomfort for the patient. The punch biopsies were processed to single cell suspensions that were sorted flow cytometrically on the basis of β_1 -integrin expression. Subsequently, growth characteristics of β_1 -integrin dim and bright cells in cell culture were determined by means of image analysis.

With this combination of flow cytometry, cell culture and image analysis we investigated whether the

β_1 -integrin dim and bright populations behave differently with respect to the number of colonies formed, start of colony formation and growth rate.

MATERIALS AND METHODS

Biopsy procedure

Residual human skin from abdominoplasty was obtained from women aged 28–65 years. Pieces of skin from about 4 cm square were kept in MEM (Eagles, 25 mm Hepes with Earle's salts, without L-glutamine) (Gibco BRL, Life Technologies, New York, USA) with 1% v/v antibiotic-antimycotic solution (Gibco) for a maximum of 8 days at 4°C. Ten punch biopsies with a diameter of 4 mm were taken from this material.

Cell isolation and labelling procedure

Biopsies were kept in phosphate buffered saline (PBS) containing 2.5 mg/ml trypsin (Difco Laboratories, Detroit, MI, USA) at 4°C for 16–20 h; 10% fetal calf serum (Harlan Sera-Lab, Loughborough, UK) was added and the dermis and epidermis were separated with tweezers. The epidermis and dermis were gently mixed on a vortex. After the stratum corneum was removed, the cells were pelleted and the supernatant was discarded. Subsequently, the pellet of cells was resuspended and labelled with 3250 μ l of K20-FITC (DAKO, Copenhagen, Denmark), an antibody directed against β_1 -integrin which was diluted 1:20. Afterwards, cells were washed with PBS/1% fetal calf serum and centrifuged for 5 min at 1000 g. After discarding the supernatant, the pellet was resuspended and the cells were sorted.

Cell sorting

The labelled cells were sorted by an EPICS[®] Elite flow cytometer (Coulter, Luton, UK), equipped with an autoclone unit. A bandpass filter of 515–535 nm (green, FITC) was used to measure emitted light. A gate in the right angle scatter (a measure of regularity) versus forward scatter (a measure of size) diagram was used to exclude debris. Gates in the FITC expression versus forward scatter diagram were used to sort the β_1 -integrin-positive cells. Control samples consisted of cell suspensions in which the primary antibody was omitted. Based upon the measurement of these controls, gates were set in such a way that if a primary antibody was omitted, the proportion of the fluorescence-positive signal for this omitted antibody was between 0% and 1%. Isotype antibody controls were performed when the staining procedure was initially set up; however, the pattern was so clear that this was omitted in further experiments (18).

The 10% of cells that were least positive were defined as the β_1 -integrin dim cells and the 10% of the cells that were most positive were classified as the bright cells (Fig. 1). This was done to avoid overlap between the subpopulations. In each experiment, a 96-well plate was filled with 150 β_1 -integrin dim cells per well and another was filled with 150 β_1 -integrin bright cells per well. This was carried out in five consecutive experiments in triplicate or (in one experiment) in duplicate.

Cell culture

Sorted keratinocytes were seeded on lethally irradiated (3000 rad, 3.2 min) Swiss mouse 3T3 fibroblasts according to the Rheinwald-Green system (19). The cells were cultured

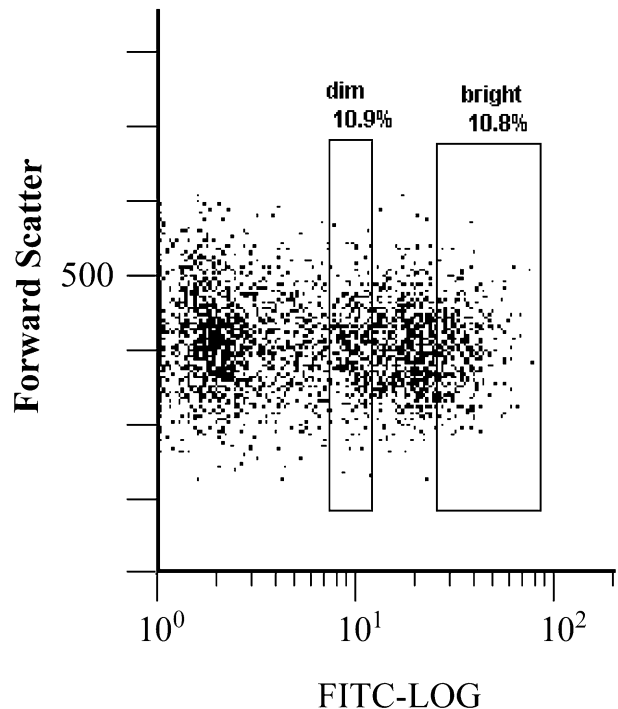


Fig. 1. Gates in the FITC expression vs forward scatter (FS) diagram were used to sort different β_1 -integrin subpopulations. The 10.9% of cells that were least positive were defined as the β_1 -integrin dim cells and the 10.8% of the cells that were most positive were classified as the bright cells.

in DMEM/F12 (3:1, v/v, Bio Whittaker, Walkersville, MD, USA) supplemented with 0.4 μ g/ml hydrocortisone (Brunschwig Chemie BV, Amsterdam, The Netherlands), 10⁻⁶ M isoproterenol (Sigma, St Louis, MO, USA), 100 i.u./ml penicillin and 100 μ g/ml streptomycin (Gibco Laboratories, Breda, The Netherlands), 6% fetal calf serum (Harlan Sera-Lab, Loughborough, UK) and 10 ng/ml epidermal growth factor (Sigma). After sorting, the 96-well plates were placed in an incubator at 37°C, 95% humidity and 8% CO₂ in air.

Image analysis

From the moment colonies were visible, they were recorded three times a week using a Zeiss (Thornwood, NY, USA) Axiovert 35M inverted microscope equipped with a 10 \times /0.3 Ph1 bright phase contrast objective connected to a 512 \times 512 video camera (HCS MX5, DIFA, Breda, The Netherlands). Signals were digitized with Scion CG-7 or Pixel Pipeline frame grabbers in a Macintosh workstation (G4 or Quadra 800). The wells of a 96-well plate were positioned with an EK8b MTP scanning table (Marzhauser, Wetzlar, Germany) manually controlled by the operator. The images of the wells were stored in a microprocessor. Scion Image for Windows beta 4.0.2 (Scion Corporation) was used to analyse the recorded images. In this program cells were manually marked by the investigator.

Statistical analysis

Regression analysis was used to relate the increase in cell size of the β_1 -integrin bright subpopulation to age; in this way colony size was converted to cell number. To compare the

number of colonies formed, an analysis of variance (ANOVA) with repeated measurements was performed.

RESULTS

All colonies that were formed in five consecutive experiments, with cells from five different donors, were followed for 28 or 29 days. The first colonies were detectable from the 10th day after seeding and from that time point all colonies were counted and recorded three times a week. At day 14 the first colonies began to reach confluence. An example of the colonies formed is shown in Fig. 2.

To determine growth characteristics, we first investigated whether cell size differed when colonies grew older. Area and cell number of representative colonies at different time points were measured. A calibration curve was made from one series of β_1 -integrin dim and one series of bright cells from each experiment. Subsequently, the relationship between cell size (i.e. area divided by number of cells in a colony) and age of a colony (i.e. number of days after seeding) was determined. For the β_1 -integrin dim subpopulation it turned out that cell size was independent of the age of the

colony. This meant that it was sufficient to measure area because the number of cells in the colony could be read from the calibration curve. In contrast, in the β_1 -integrin bright subpopulation cell size increased when colonies became older. For that reason a formula was created that comprises a factor that corrects for age of the β_1 -integrin bright colony when area was converted into cell number.

We found that the number of colonies in the β_1 -integrin dim subpopulation exceeded that in the bright subpopulation (ANOVA, repeated measurements, revealed a borderline significance, $p=0.05$). In both groups the number of colonies increased until confluence was reached; from that moment on some colonies were indistinguishable from each other and were counted as one. In Fig. 3A a typical example of the number of colonies in the β_1 -integrin bright and dim subpopulation is shown, whereas Fig. 3B is an overview of all experiments presenting the number of colonies formed 15 days after seeding in the β_1 -integrin dim and bright subpopulation on a logarithmic scale.

Before growth characteristics could be determined, all useful colonies were selected: colonies that were

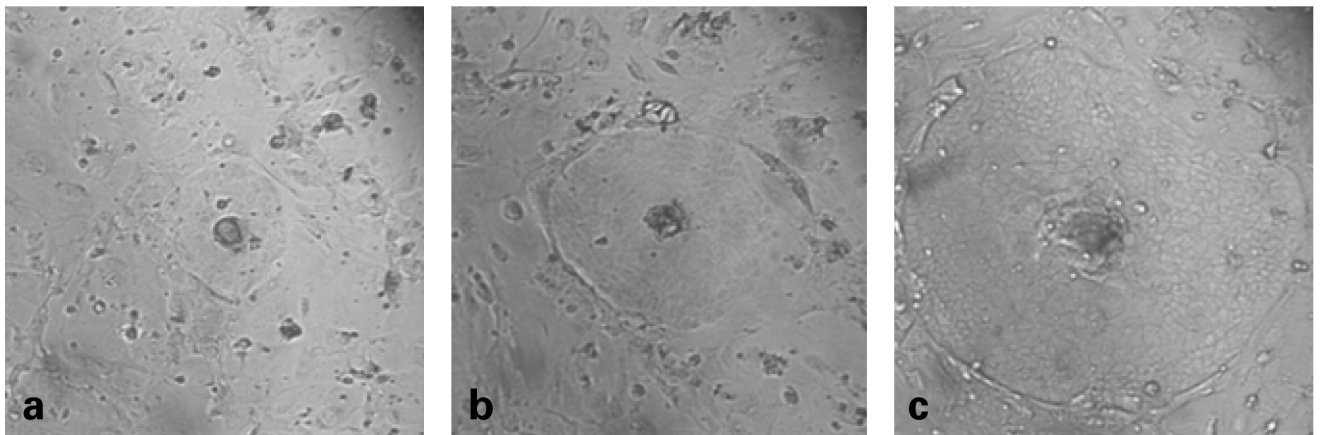


Fig. 2. Example of a small colony that enlarges.

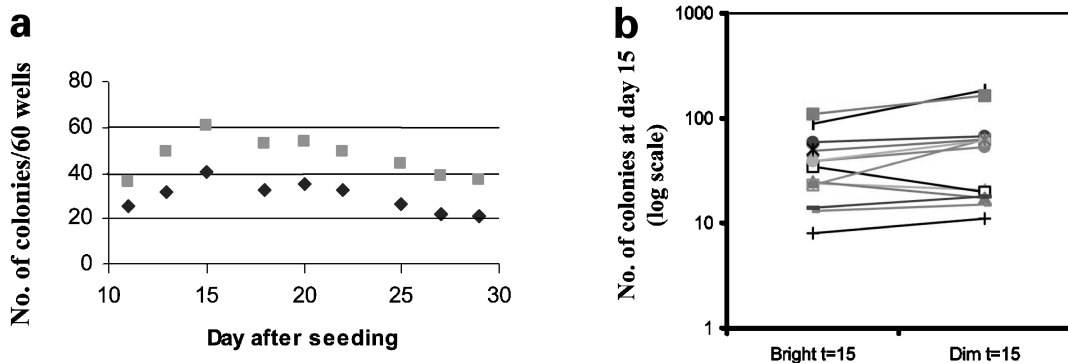


Fig. 3. (a) A typical example which illustrates that the mean number of colonies formed in the β_1 -integrin dim subpopulation (squares) exceeds that in the bright subpopulation (diamonds). (b) An overview of all experiments presenting the number of colonies formed in the β_1 -integrin dim and bright subpopulations 15 days after seeding on a logarithmic scale.

recorded only once and those that touched each other or the rim were eliminated. Thus, 199 colonies in the β_1 -integrin dim subpopulation and 222 colonies in the β_1 -integrin bright subpopulation were available for analysis. First the moment of start of colony formation was compared in both subpopulations. When we compared the moment at which colonies consisted of 100 cells no differences were found between colonies in the dim and bright subpopulation.

As a measure of growth rate the increase in the number of cells of a colony divided by the time in which the increase took place was determined. Statistical analysis showed that colonies in the β_1 -integrin dim subpopulation grew significantly faster than those in the bright subpopulation (t-test, $p=0.008$). An example of growth curves of β_1 -integrin dim and bright cells is shown in Fig. 4.

DISCUSSION

In the present study, phenotypic and functional differences between β_1 -integrin bright and dim cells were determined with the use of flow cytometry, cell culture and image analysis. With a combination of these methods it was shown that cell size increased with age in the β_1 -integrin bright subpopulation. Furthermore, the number of colonies formed and their growth rate in the β_1 -integrin dim subpopulation exceeded that in the bright subpopulation. With respect to start of colony formation no differences were found.

A remarkable finding for colonies in the β_1 -integrin bright subpopulation, which harbours the putative stem cells, was that cell size increased when colonies aged. This was different from results in another study in which single unfractionated keratinocytes were inoculated and which showed a constant cell size over a large range of colony sizes (15). The difference in our study can be explained by the difference in age between TAC in the β_1 -integrin bright subpopulation (which are

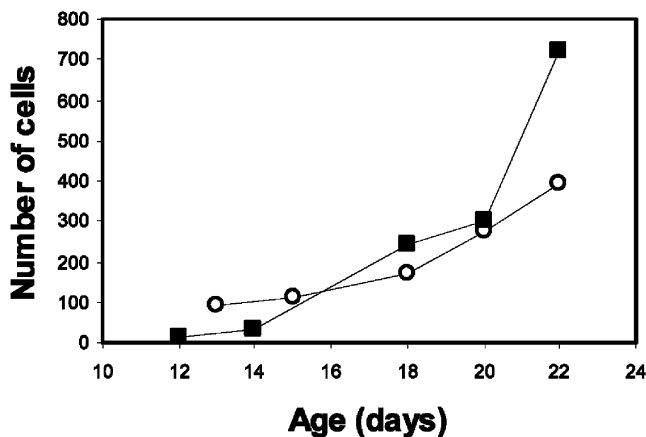


Fig. 4. Representative growth curves of the β_1 -integrin dim cells (■) and bright cells (○).

young TAC derived from ESC that were initially seeded) and those in the β_1 -integrin dim subpopulation (which were already older TAC when they were seeded). It is known that cells in the basal compartment, which comprises the putative ESC, are small and that the suprabasal ones progressively enlarge when they move upwards and differentiate. This means that young TAC in the β_1 -integrin bright subpopulation enlarge when they become older (13, 20), whereas the older TAC in the dim subpopulation will not considerably increase in size.

The number of colonies in the β_1 -integrin dim subpopulation exceeded that in the bright subpopulation. This can be explained by the difference in cell cycle time between ESC and TAC: it will take longer before the first TAC arises from a slowly cycling ESC than from the TAC that was seeded in the β_1 -integrin dim subpopulation. This means that the TAC in the dim subpopulation could divide more frequently and form more colonies than TAC that are derived from ESC in the span of time of this experiment. An earlier study showed that cells with the strongest expression of β_1 -integrin had a higher colony-forming efficiency after 14 days than cells with a low level of β_1 -integrin (3).

With respect to the start of colony formation no differences were found between colonies in the β_1 -integrin dim and bright subpopulation. This could be because it was impossible to detect colonies at an early stage: until day 10 we were not able to detect colonies by bright field microscopy. The first colonies that were detected already comprised at least 20 cells. As TAC have a shorter cell cycle time, the period between seeding of the cells and the first cell division must be shorter than that for the ESC in the β_1 -integrin bright subpopulation. Therefore, colony formation in the β_1 -integrin dim subpopulation is expected to start earlier than that in the bright subpopulation. In order to detect small colonies earlier, we labelled them with fluorescent dyes that are suitable for viable cells. SYTO 13, a cell-permeant nucleic acid stain, and carboxy-fluorescein diacetate succinimidyl ester (CFSE), an intracellular covalently coupling dye, were tested. A clear distinction between small colonies – which were hardly detectable by bright-field microscopy – and feeder cells was present (Fig. 5). SYTO13 has been described as a useful dye for other cell types (21), but it turned out that keratinocytes could not stand the required concentration. In studies with lymphocytes the use of CFSE has been described as a dye that allows long-term tracking of lymphocytes (22–24). However, we did not succeed in labelling keratinocytes with this dye.

When growth rates were compared, it was found that colonies in the β_1 -integrin dim subpopulation grew faster than those in the bright subpopulation. This could be explained by the presence of ESC, with a long

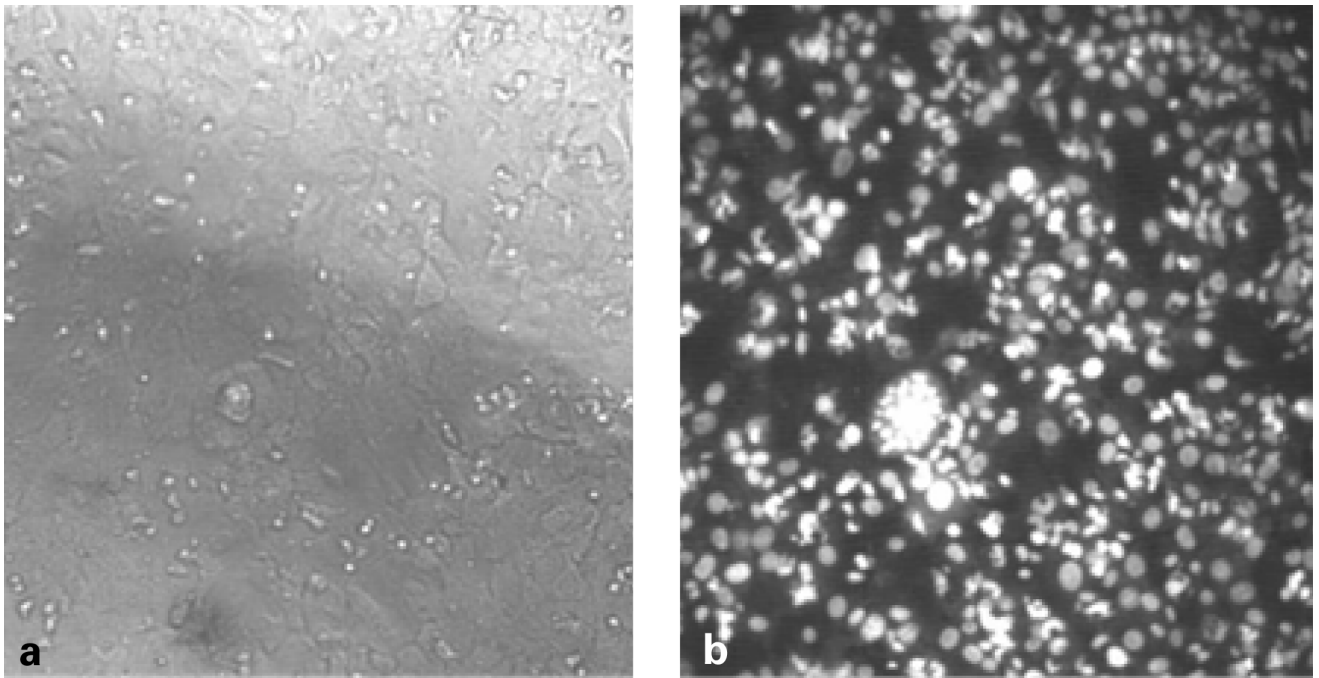


Fig. 5. (a) A small colony that is hardly distinguishable from the surrounding feeder cells. (b) The same colony labelled with SYTO 13, which stains nucleic acid in viable cells.

cell cycle time, in the β_1 -integrin bright subpopulation. Although the predominant replicating cell in tissue as well as in culture is the TAC (14), it takes longer in the β_1 -integrin bright subpopulation until the first TAC arises from a ESC than for the first TAC to arise from another TAC in the β_1 -integrin dim subpopulation. The presence of ESC in the β_1 -integrin bright subpopulation increases the mean cell cycle time and therefore decreases the mean growth rate of the colonies.

The short duration of the experiment, 28 or 29 days, imposes a restriction on this study. This also applies to the 96-well plates that were used: because of the small surface of a well, confluence or contact inhibition was reached early. As ESC have an unlimited proliferative potential, the number of colonies in the β_1 -integrin bright subpopulation is expected to exceed that in the dim subpopulation when time and space are not limiting factors.

There is no consensus on the use of β_1 -integrin as a marker to distinguish TAC and ESC: some studies state that a two- to three-fold difference in integrin levels is present on the surface of ESC and TAC (1, 12). In contrast, others report that both ESC and TAC express high levels of β_1 -integrin (8). From earlier studies it is known that 25–50% of the basal cells exhibit high β_1 -integrin expression (12, 25, 26). As maximally 10% of all basal cells are supposed to be ESC (8, 12, 25–30), it can be concluded that only a fraction of the β_1 -integrin bright cells are ESC. This conclusion is supported by the finding that only 25% of the keratinocytes in integrin-bright patches in foreskin are clonogenic (31). For that reason a further refinement of the technique

used, by combining β_1 -integrin with other potential ESC markers, will be useful for a narrower definition of putative ESC.

Selective sorting and culturing of keratinocytes forms the basis for studies on the role of epidermal ESC with respect to therapeutic intervention and gene therapy. The application of small punch biopsies allows use of human skin with only minimal inconvenience for the patient in the future. The present study shows that a combination of flow cytometry, cell culture and image analysis offers the opportunity to characterize subpopulations of epidermal human keratinocytes phenotypically as well as functionally. Our findings support the concept that the β_1 -integrin bright fraction harbours the putative ESC and that the β_1 -integrin dim cells contain predominantly TAC.

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