Phenotypical and Functional Differences in Germinative Subpopulations Derived from Normal and Psoriatic Epidermis

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A model that explains how maintenance of normal homeostasis in human epidermis is achieved describes a heterogeneous cell population of stem cells (SC) and transit amplifying cells (TAC). There must be a tightly regulated balance between SC self-renewal and the generation of TAC that undergo a limited number of divisions before giving rise to postmitotic, terminally differentiated cells. To investigate whether this balance is disturbed in psoriatic epidermis, we have characterized flow sorted enriched SC and TAC using immunocytochemistry, flow cytometry, and real-time quantitative PCR. Our data demonstrate phenotypical and functional differences in SC (β_1-integrin bright) and TAC (β_1-integrin dim) enriched fractions between normal and psoriatic keratinocytes. Some of these were expected, such as mRNA levels of keratins 6 and 10 and of the Ki-67 antigen. Most remarkable were differences in phenotype of the psoriatic TAC compared with TAC from normal skin. These subpopulations also displayed striking differences following culture. Downregulation of markers associated with the regenerative phenotype (psoriasin, elafin, psoriasis-associated fatty acid binding protein) in cultured psoriatic dim cells in the absence of hyperproliferation suggests that proliferation and regenerative maturation are coupled. From these results, in combination with our earlier findings, we propose a model for epidermal growth control in which TAC play a crucial role.

Key words: cell culture/epidermis/proliferation/psoriasis/stem cells/transit amplifying cells
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The epidermis is a stratified squamous epithelium, which is under a constant state of proliferation, terminal differentiation, and elimination. In this way the functional integrity of the tissue is sustained. The intact epidermis has the ability to respond to a diversity of environmental stimuli, and by continuous turnover it can maintain its normal homeostasis. The processes involved and the mechanisms by which the epidermal stem cells (SC) differentiate are largely unknown. An accepted model that explains how maintenance of normal homeostasis is achieved describes a heterogeneous germinative cell population of SC and more differentiated transit amplifying cells (TAC) (Jones *et al*, 1995; Jones, 1997; Watt, 2002).

Using fluorescence activated cell sorting, basal cells can be sorted into SC-enriched fractions, which strongly express β_1-integrins (bright cells) and fractions enriched for TAC that display only a weak β_1-integrin expression (dim cells) (Jones and Watt, 1993; Jones *et al*, 1995; Jones, 1997). Apart from the β_1-integrin family, several markers have been suggested as indicative for the epidermal SC or the TAC (reviewed by Potten and Booth, 2002). It was shown that the transcription factor p63 was abundantly expressed in holoclones, which are believed to contain SC, whereas it was greatly reduced in meroclones that are formed by "young" TAC and undetectable in paraclones consisting of terminal colonies prone to differentiate (Pellegrini *et al*, 2001). The expression pattern of the α6-integrin has revealed no differences between SC and TAC, but combining the α6-integrin expression with the transferrin receptor (10G7 antigen, later known as CD71) expression, which was indicative for TAC, SC and TAC can be distinguished as $\alpha_6^{bright}10G7^{dim}$ and $\alpha_6^{bright}10G7^{bright}$ cells, respectively (Li *et al*, 1998; Tani *et al*, 2000). Furthermore, using proteomics on β_1-integrin bright and dim cells, the psoriasis-associated fatty acid binding protein (PA-FABP) was found to be upregulated in the transit amplifying fraction (O'Shaughnessy *et al*, 2000).

Although several markers have been proposed, the keratinocyte SC and its daughter, the TAC are still poorly defined. One function of the TAC is to amplify the number of cells derived from asymmetric cell division by SC to allow sufficient cell production for terminal differentiation and formation of skin barrier. In this way, SC can divide infrequently and still the terminal differentiation compartment is replenished. It has always been assumed that SC when not dividing were held in the G_0/G_1 phase of the cell cycle, but no direct evidence to prove this hypothesis has been published, partly because isolating a pure isolation of SC was problematic (Dunnwald *et al*, 2003). With respect to the TAC

Abbreviations: FITC, fluorescein isothiocyanate; PA-FABP, psoriasis-associated fatty acid binding protein; SC, stem cell; TAC, transit amplifying cell

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of the epidermis, research is even more limited. It is generally accepted that as soon as cells leave the SC compartment, they are committed to terminal differentiation. This process is considered to be continuous and irreversible.

In normal epidermis, the majority of basal germinative cells are quiescent and only a small fraction is proliferating. In psoriasis, a chronic skin disease of yet unknown etiology, which is characterized by epidermal hyperproliferation and regenerative maturation (Mansbridge *et al*, 1984), most germinative cells are proliferating. This regenerative phenotype has been thoroughly studied previously and includes the expression of the differentiation markers keratin (K) 6, K16 (Leigh *et al*, 1995), psoriasin (Madsen *et al*, 1991), elafin (Schalkwijk *et al*, 1990), and PA-FABP (Madsen *et al*, 1992; Masouye *et al*, 1996; Watanabe *et al*, 1997). It is generally estimated that SC constitute between 1% and 10% of the basal layer (Potten and Morris, 1988), and as approximately 40% of the basal layer exhibits high levels of β_1-integrin (Jones *et al*, 1995), it is likely that these basal cells contain a small number of SC and a significant number of TAC. In fact, in psoriasis the β_1-integrin expression is increased and β_1-integrin-positive cells are also seen in the suprabasal layers (Hertle *et al*, 1992; Watt and Jones, 1993). Using mathematical models, the typical psoriatic histology was explained as a result of a markedly increased proliferative compartment (Watt and Hertle, 1994; Iizuka *et al*, 1996; Iizuka *et al*, 1997). The mechanisms that drive the keratinocyte hyperproliferation in psoriasis are largely unknown. It is not known whether the defect in growth control is at the level of the SC or the TAC, or both. In lesional psoriatic epidermis, a large number of cells are cycling both in the basal and the first suprabasal layers. Clearly, the TAC compartment has expanded enormously in psoriasis, giving rise to a huge cell production rate and the ensuing scaling process. A number of antipsoriatic therapies are directed at suppressing keratinocyte growth, e.g., directly, or indirectly by acting at the level of the T cell, but it is not known how antipsoriatic agents exert their antiproliferative effects. They could act at the level of the SC by suppressing the generation of daughter cells, or alternatively they could act at the level of the TAC. Even at this point several possibilities exist: antipsoriatic agents could drive basal TAC to the G_0 phase of the cell cycle by interfering with a vicious (autocrine) loop, or inhibit proliferation of late TAC in the suprabasal layer, or promote normal differentiation of the suprabasal compartment. Clearly, a better knowledge on the heterogeneity and functional characteristics of germinative subpopulations in the psoriatic epidermis is required to understand the basic abnormalities of the disease, and the mode of action of antipsoriatic agents.

On the basis of previous studies, we hypothesized that early cell kinetic changes observed in the TAC population in healing or exacerbating hyperproliferative skin are of pathogenic significance (Rijzewijk *et al*, 1992; Castelijns *et al*, 1999; Franssen *et al*, 2004). The aim of this study was to extend these investigations and compare flow-sorted β_1-integrin dim (assumed transit amplifying) cells and β_1-integrin bright (putative stem) cells from normal and psoriatic skin. Using real-time quantitative (qPCR), we analyzed mRNA levels of β_1-integrin dim and bright cells that were derived from normal and psoriatic epidermis by flow sorting of freshly isolated cells. To establish whether these subpopulations differ with respect to growth behavior and colony morphology, and whether cellular characteristics are maintained *in vitro*, the β_1-integrin dim and bright subpopulations from normal and psoriatic epidermis were cultured and analyzed similarly at the protein and mRNA level. From these results, in combination with our earlier findings, we propose a model for epidermal growth in which TAC play a crucial role.

Results

Characterization of flow-sorted epidermal subpopulations from normal and psoriatic epidermis by qPCR Several studies have previously described a different protein or gene expression profile in β_1-integrin bright and dim fractions of normal human epidermis. Here, we have examined flow sorted β_1-integrin bright and dim fractions from psoriatic epidermis, and compared these with the subpopulations in normal epidermis using qPCR. Although the expression pattern of β_1-integrin at the protein level allows a clear separation between bright and dim keratinocytes (Fig 1), normal keratinocytes revealed only a slightly elevated mRNA level of β_1-integrin in the bright keratinocytes, whereas there was a 2-fold elevated mRNA level in psoriatic bright keratinocytes compared with the dim fraction ($p < 0.05$) (Fig 2*A*).

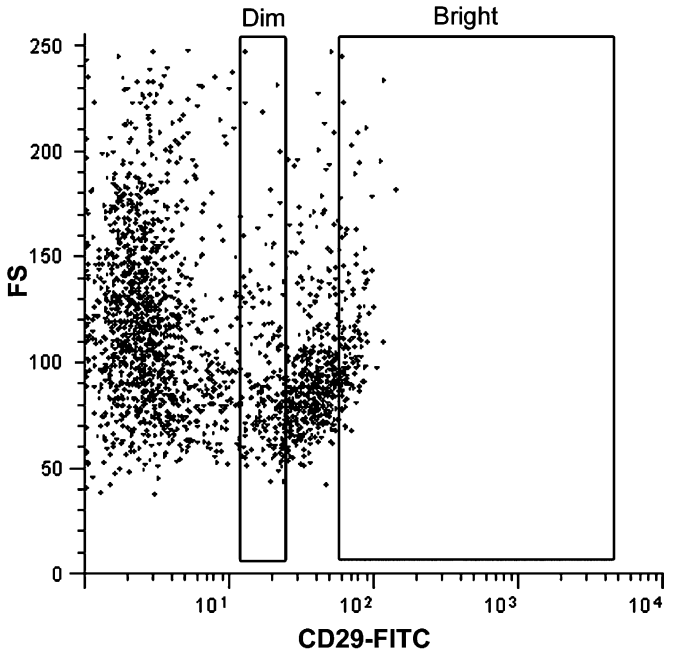


Figure 1
Flow sorting of β_1-integrin-positive subpopulations. As depicted, a dot plot representing cell size (FS)/CD29-fluorescein isothiocyanate (FITC) fluorescence per cell allows a clear separation between the β_1-integrin-negative and the β_1-integrin-positive population. Based on the mean CD29-FITC fluorescence, gates can then be set in the β_1-integrin-positive population; approximately 10% of the cells with the highest β_1-integrin expression are defined as bright cells and 10% of the cells with the lowest β_1-integrin expression are identified as dim cells. In this way, it was possible to prevent overlap between both subpopulations, and rather selective subpopulations of each cell type were obtained for further experiments.

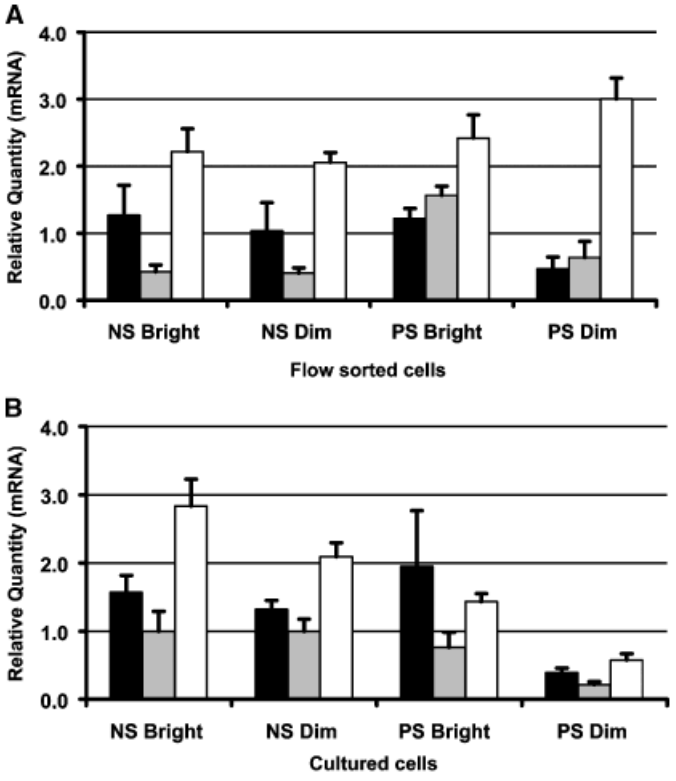


Figure 2
Integrin mRNA expression in germinative epidermal subpopulations. mRNA quantitation (y-axis) was obtained relative to the reference gene human acidic ribosomal protein P0 according to the method of Livak (Livak and Schmittgen, 2001). (*A*) mRNA levels of β_1- (*black bars*), α_6- (*gray bars*), and β_4-integrin (*white bars*) in flow-sorted fractions of bright and dim cells obtained from normal and psoriatic epidermis. (*B*) mRNA levels of β_1-, α_6-, and β_4-integrin in cultured bright and dim cells derived from normal and psoriatic epidermis. Note the low levels of expression of all integrins in cells cultured from psoriatic dim cells. Results of sorted cells represent mean values $\pm$ SEM of three different patients, whereas cultured cells were based on four samples. NS, normal skin; PS, psoriatic skin.

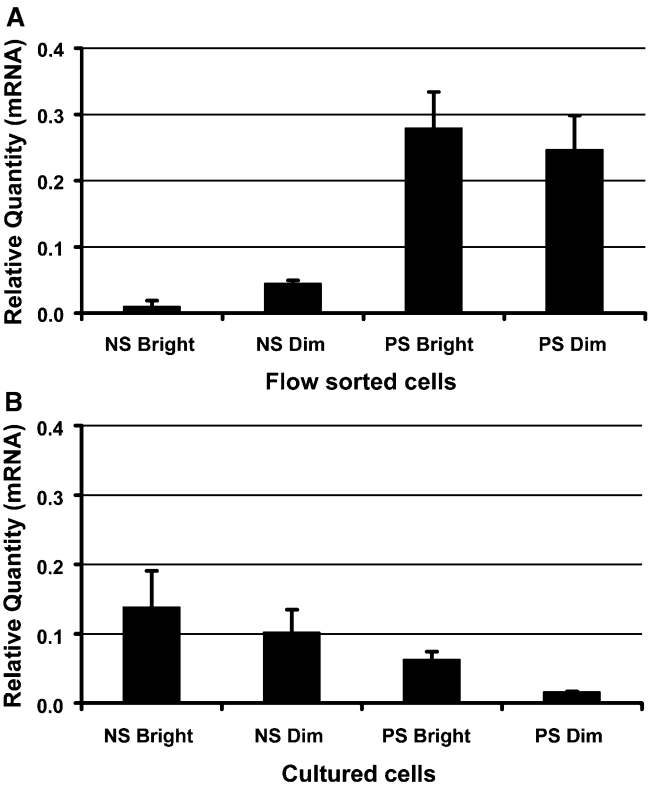


Figure 3
Ki-67 mRNA expression in germinative epidermal subpopulations. mRNA quantitation (y-axis) was obtained relative to the reference gene human acidic ribosomal protein P0 according to the method of Livak (Livak and Schmittgen, 2001). (*A*) Ki-67 levels (*black bars*) were nearly undetectable in flow-sorted normal cells; substantially higher levels were found in psoriatic cells. (*B*) *In vitro,* Ki-67 levels showed a strong increase in normal cells compared with freshly isolated cells; psoriatic bright and dim cells showed a strong decrease compared with freshly isolated cells. Results of sorted cells represent mean values $\pm$ SEM of three different patients, whereas cultured cells were based on four samples. NS, normal skin; PS, psoriatic skin.

mRNA levels of α_6-integrin, which is the partner subunit for β_4-integrin in the hemidesmosome, were similar for both the normal β_1-integrin bright and dim keratinocytes, whereas the psoriatic β_1-integrin bright cells showed 3-fold higher levels compared to the psoriatic dim keratinocytes ($p < 0.05$) (Fig 2*A*). No differences in β_4-integrin mRNA levels were found between bright and dim cells, irrespective of the tissue from which they were obtained (Fig 2*A*). mRNA levels of the epidermal differentiation marker K10 were higher in the β_1-integrin dim fraction of both normal (2-fold) and psoriatic (7-fold, $p < 0.01$) keratinocytes compared with the bright fractions. K6 mRNA levels were, as expected, virtually undetectable in both fractions of normal keratinocytes, whereas both psoriatic β_1-integrin bright and dim keratinocytes showed elevated levels of K6 mRNA ($p < 0.001$). Again, there was a major difference between psoriatic β_1-integrin bright and dim cells, with a 3-fold higher mRNA level in the psoriatic dim cells ($p < 0.001$, data not shown). As a measure of proliferative activity, the Ki-67 mRNA levels were examined, and as expected, these were relatively low in normal β_1-integrin bright and dim keratinocytes although the level in the dim cells was 3-fold higher than in the bright cells ($p < 0.05$). In psoriatic keratinocytes, a 5-fold higher level of Ki-67 mRNA was seen in both the dim and bright fraction compared with the normal keratinocytes ($p < 0.05$) (Fig 3*A*). Although the transferrin receptor (CD71) has been suggested as a marker for TAC, in our hands, mRNA levels were almost identical in normal β_1-integrin bright and dim keratinocytes, with slightly elevated levels in both the psoriatic fractions compared with the normal subpopulations (Fig 4*A*). Remarkably, the mRNA levels of the putative SC marker p63 revealed a 2-fold higher level in normal β_1-integrin bright cells compared with normal β_1-integrin dim cells, whereas no differences were found between the psoriatic dim and bright keratinocytes. Surprisingly, mRNA levels for collagen VII revealed a similar trend as the p63 levels, with an even stronger difference between the levels measured in β_1-integrin bright keratinocytes compared with β_1-integrin dim keratinocytes for both normal (NS) and psoriatic skin ($p < 0.05$) (Fig 4*A*). Finally, we analyzed a number of markers that are associated with abnormal differentiation in psoriatic epidermis. These include elafin and psoriasin, which are not expressed in normal skin, and PA-FABP, which shows a restricted (stratum granulosum) expression in normal skin. In the upper suprabasal layers of psoriatic epidermis, high expression levels of elafin, psoria-

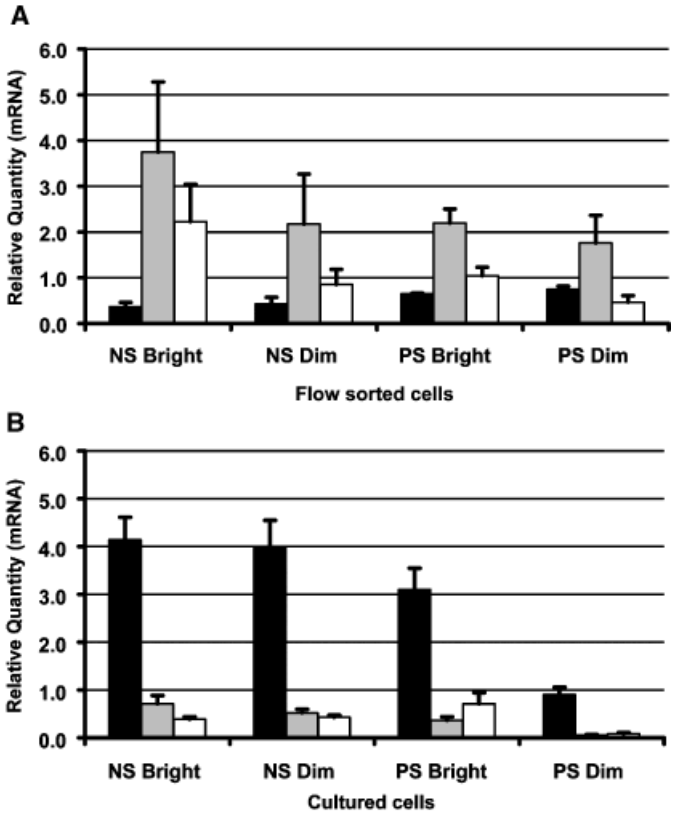


Figure 4
CD71, collagen type VII, and p63 mRNA expression in germinative cell populations. mRNA quantitation (y-axis) was obtained relative to the reference gene human acidic ribosomal protein P0 according to the method of Livak (Livak and Schmittgen, 2001). (*A*) CD71, a putative transit amplifying cell marker (*black bars*) and p63, a putative stem cell marker (*gray bars*), did not provide a clear discrimination of β_1-integrin bright and dim cells sorted from normal and psoriatic epidermis. Collagen VII (*white bars*), however, was consistently higher in β_1-integrin bright cells compared with dim cells. (*B*) CD71 is strongly upregulated in cultured cells. All three markers show low expression levels in cultured psoriatic dim cells compared with the other cultured cell populations. Results of sorted cells represent mean values $\pm$ SEM of three different patients, whereas cultured cells were based on four samples. NS, normal skin; PS, psoriatic skin.

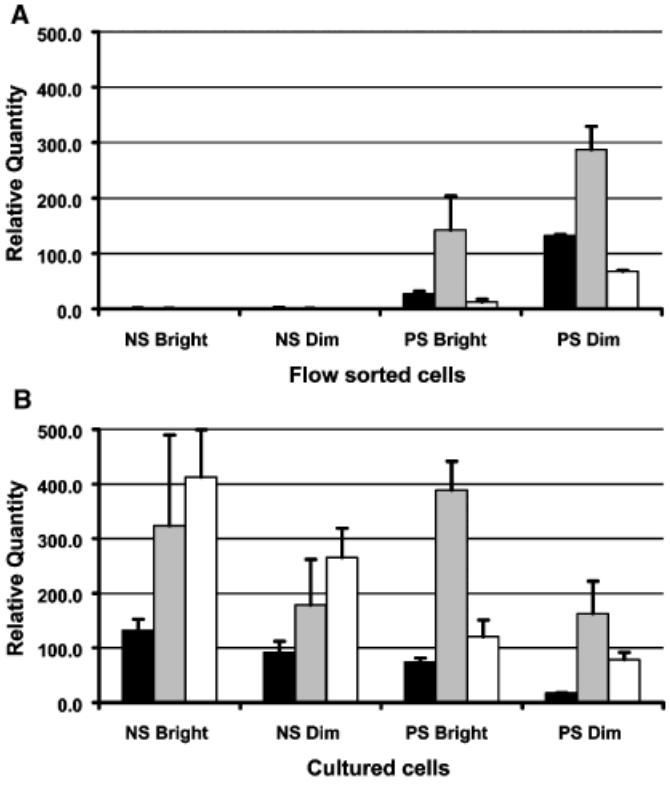


Figure 5
Elafin, psoriasin, and psoriasis-associated fatty acid binding protein (PA-FABP) mRNA expression in germinative epidermal subpopulations. mRNA quantitation (y-axis) was obtained relative to the reference gene human acidic ribosomal protein P0 according to the method of Livak (Livak and Schmittgen, 2001). (*A*) mRNA levels of PA-FABP (*black bars*), psoriasin (*gray bars*), and elafin (*white bars*) in flow-sorted fractions of bright and dim cells were virtually undetectable in keratinocytes derived from normal skin, and were high in the dim fractions derived from psoriatic skin. (*B*) *In vitro,* a strong increase of all three transcripts was found in cultured normal keratinocytes compared with flow-sorted cells. In cultured psoriatic dim cells, PA-FABP and psoriasin were downregulated compared with flow-sorted cells. Elafin expression was remarkably lower in cultured psoriatic cells compared with cultured normal cells. Results of sorted cells represent mean values $\pm$ SEM of three different patients, whereas cultured cells were based on four samples. NS, normal skin; PS, psoriatic skin.

sin, and PA-FABP are found. As expected, normal keratinocytes revealed no detectable mRNA levels of these transcripts, whereas expression levels in psoriatic keratinocytes revealed a strong expression of PA-FABP ($p < 0.01$, 5-fold), psoriasin (2-fold), and skin-derived anti leucoprotease (SKALP)/elafin ($p < 0.01$, 5–6-fold) in the psoriatic β_1-integrin dim keratinocytes compared with the psoriatic bright cells (Fig 5*A*). These findings suggest that transcripts associated with regenerative maturation are already present in the TAC population. Additionally, the expression levels of transcripts involved in cell cycle control were examined, including the Cdk inhibitory proteins p16, p21, and p27, which are believed to suppress G_1/S Cdk activity, and maspin, a recently discovered tumor suppressor that is believed to be upregulated in keratinocyte senescence. To establish whether these transcripts were already upregulated in the β_1-integrin bright and dim subpopulations, the expression levels of p16, p21, p27, and maspin were examined, and as expected they were low in all fractions immediately after sorting (Fig 8*A*).

Phenotypical characterization of keratinocytes cultured from normal and psoriatic skin To examine whether differences found in freshly isolated keratinocytes are maintained in culture, and whether these are associated with different growth behavior and morphology, β_1-integrin bright and dim keratinocytes were flow sorted and seeded on a feeder layer of 3T3 cells that supports colony formation (Rheinwald and Green, 1975). Approximately 6 d after seeding, the first colonies could be detected in the β_1-integrin bright and dim cultures of normal keratinocytes, whereas the cultures from psoriatic keratinocytes did not show any colonies until day 8 or 9 after seeding. In normal keratinocytes, a subconfluence of 60% was reached after approximately 15 d, whereas confluence was reached after 22 d. On the other hand, psoriatic cultures did not reach 60% confluence until approximately 22 d and confluence at 29 d after seeding. Remarkably, confluency was only achieved in the cultures derived from the psoriatic β_1-integrin bright keratinocytes, whereas cultures derived from psoriatic β_1-integrin dim keratinocytes showed

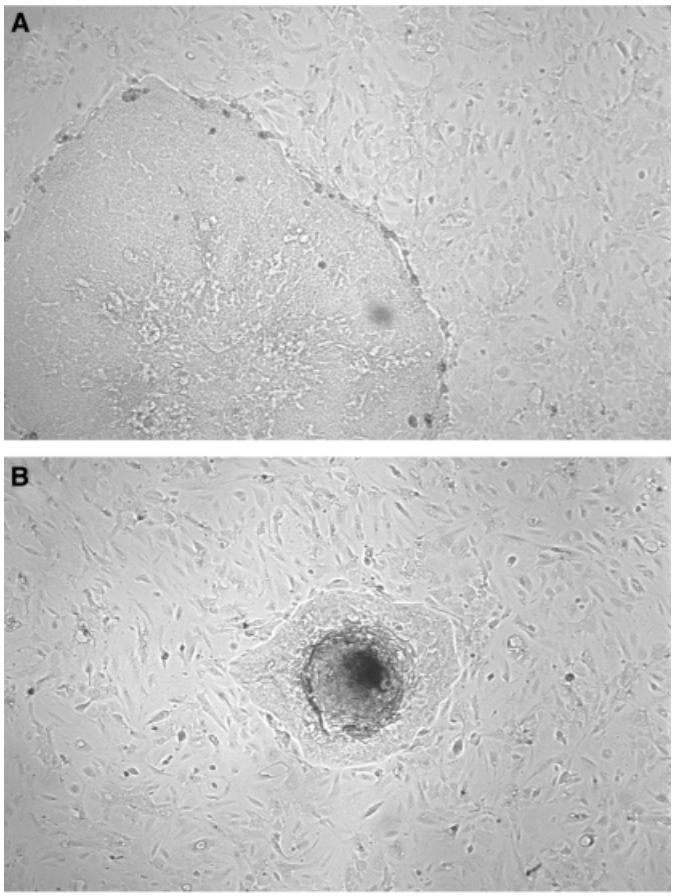


Figure 6
Morphology of keratinocyte colonies. Seventeen days after seeding, the psoriatic bright cultures consisted of large colonies with minimal keratinization that reached confluency within 1 mo (*A*); cultures of psoriatic dim cells showed, however, predominantly small abortive colonies with a markedly keratinized center (*B*). Similar differences were found less frequently in cultures derived from normal skin (data not shown).

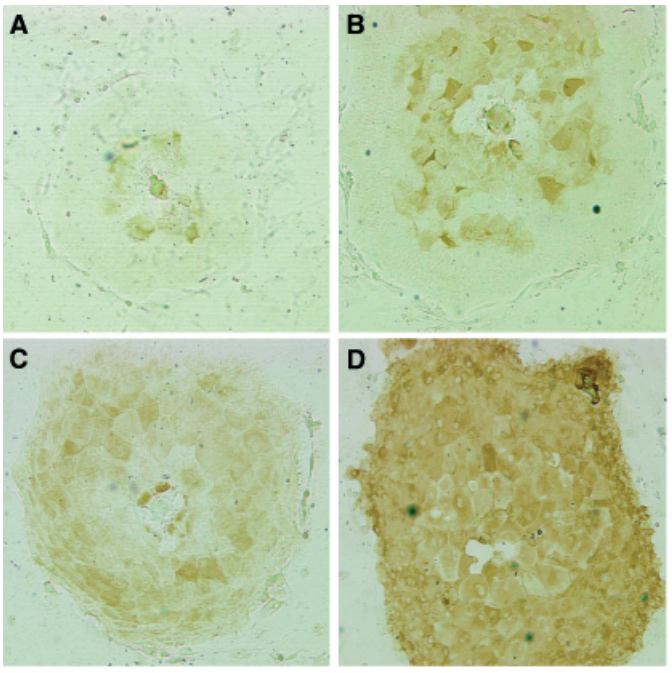


Figure 7
Immunocytochemical staining of cultured keratinocytes. Immunocytochemical staining of normal keratinocytes cultured on coverslips revealed differences in keratin (K) 10 expression (*A* and *B*) and K6 expression (*C* and *D*) between the β_1-integrin bright (*A* and *C*) and the β_1-integrin dim (*B* and *D*) fraction. Notice the strong K10 and K6 expression in the dim fraction (similar staining patterns were observed in psoriatic cultures; data not shown).

predominantly small abortive colonies (presumed paraclones) with a pronounced keratinization in the center of the colony (Fig 6). The β_1-integrin bright colonies revealed considerably less keratinization and showed an increase in size over time until confluency was reached. The psoriatic β_1-integrin dim colonies did not increase in size any further and neither did they reach confluency, not even after 29 d. This phenomenon was rarely observed in cultures of normal keratinocytes.

For cytochemical analysis, we seeded flow-sorted keratinocytes on coverslips and the cells were cultured as indicated above. Subsequently, staining for a number of integrins and differentiation markers was performed. Figure 7 shows cytokeratin 10 and 6 expression in cultured normal keratinocytes at 14 d after seeding. A marked difference was noted between dim and bright cells, both in normal and psoriatic skin (data not shown). For most markers, however, quantification of positive cell numbers and staining intensity was virtually impossible. To enable quantification, we used multiparameter flow cytometry on cell suspensions obtained from cultures mentioned above. Cells were grown until approximately 60% and 100% confluency and subsequently harvested and stained for flow cytometric analysis. Independent of their origin, all cells in suspension were similar with respect to cell size and shape (flow cytometric forward scatter and side scatter, respectively). Furthermore, no striking differences in protein expression were found between 60% and 100% confluency. *In vitro* normal keratinocytes nearly all express the β_1-, β_4-, and α_6-integrin, the Ki-67 antigen, and the transferrin receptor, indicating a strong upregulation of these proteins in cultured cells. But, only 20%–30% of the psoriatic dim keratinocytes expressed these proteins, whereas the bright fraction showed intermediate percentages (data not shown). The number of cells expressing the differentiation markers K10 and K6 remained relatively low under the culture conditions used. Nevertheless, the expression of these keratins appeared consistently higher in cultures derived from β_1-integrin dim cells compared with cultures derived from β_1-integrin bright cells, which is in accordance with the cytochemical findings (see Fig 7). Collagen VII and p63 expression were also strongly downregulated under the conditions used, hindering an accurate quantification (data not shown).

Finally, we examined a number of genes at the mRNA level in cultured β_1-integrin bright and dim cells from normal and psoriatic epidermis as described above. The normalized expression levels are shown in Figs 2*B*, 3*B*, 4*B*, and 5*B* and can be used for comparison with the expression levels in freshly isolated cells (shown in Figs 2*A*, 3*A*, 4*A*, and 5*A*). The differences in β_1-integrin mRNA levels in the cells that were sorted on the basis of β_1-integrin immunostaining

were similar for freshly isolated and cultured keratinocytes. Keratinocytes from the β_1-integrin bright fraction in psoriatic epidermis, however, showed a marked increase compared with the freshly isolated cells. Figures 2*B* and 3*B* show that cells cultured from the dim fraction of psoriatic epidermis express significantly less α_6-integrin, β_4-integrin ($p < 0.05$), and Ki-67 ($p < 0.05$), compared with freshly isolated dim cells from psoriatic lesions. This is in contrast to the dim cell population cultured from normal skin, which showed an increase of α_6-integrin ($p < 0.05$), CD71 ($p < 0.01$), and Ki-67 (Figs 2*B*, 3*B*, and 4*B*). The cell populations cultured from bright cells of normal skin and psoriatic skin showed similar expression profiles. When compared with the freshly isolated cells, a strong increase of CD71 was noted in both the cultured bright cell populations ($p < 0.01$). The putative SC marker p63, on the other hand, showed a strong decrease in all cultured subpopulations, irrespective of the subpopulation and tissue from which they were cultured. A similar phenomenon was noticed for the collagen VII mRNA levels (Fig 4*B*), and the marker for epidermal differentiation K10 (data not shown). The mRNA levels of K6 showed a strong decrease in the dim cells cultured from psoriatic skin, whereas an unchanged level was noted in the psoriatic bright cells. The cell populations cultured from normal skin showed a strong increase of K6 under these culture conditions (data not shown). A marked difference was seen with respect to Ki-67 expression; cultured bright cells from normal skin showed a strong increase, whereas a strong decrease of Ki-67 was noted in cultured bright cells from psoriatic skin, when compared with the expression levels in freshly isolated cells (Fig 3*A*, *B*). These data reveal a substantial difference between germinative cell populations from normal and psoriatic skin.

Figure 5*A*, *B* shows the expression levels of genes that are associated with aberrant differentiation in psoriasis (elafin, psoriasin, PA-FABP). A strong induction of all three markers was found in both cell populations cultured from normal skin, indicating the well-known phenomenon that keratinocytes from normal skin can acquire the regenerative maturation phenotype in cell culture. Remarkably, only a modest induction was seen in psoriatic bright cells, and a decrease in expression of psoriasin (NS) and PA-FABP ($p < 0.01$) was noted in the dim cells. Again, this indicates the marked differences between the germinative cell populations of normal and psoriatic epidermis. Additionally, the expression levels of p16, p21, p27, and maspin were examined in the cultured subpopulations. The low expression levels of p21 did not change appreciably during the culture period. Maspin showed no significant differences in the expression levels in sorted cells and again not in the cultured subpopulations. The expression levels of p27 (NS) showed a marked increase in the cultured subpopulations; however, no significant differences in expression were noticed between the β_1-integrin bright and dim fractions of both normal skin and psoriatic skin. The expression level of p16 showed an increase in the cultured fractions of normal and psoriatic cells (Fig 8*A*, *B*), with a strong increase in the expression levels measured in the bright fraction of both normal ($p < 0.01$) and psoriatic ($p < 0.01$) skin. But, significant differences were only noted between the bright and dim fraction of psoriatic skin ($p < 0.05$).

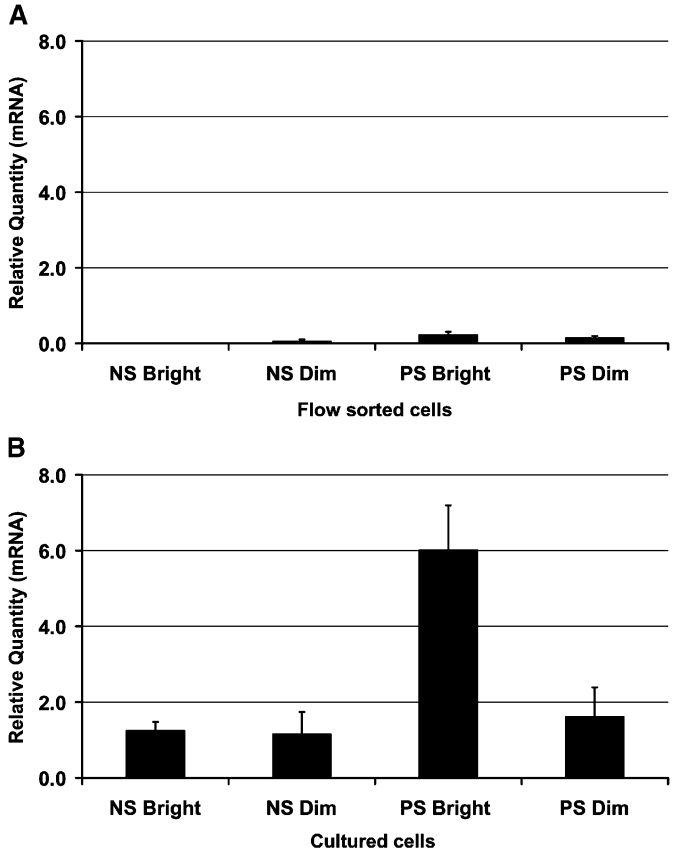


Figure 8
p16 mRNA expression in germinative epidermal subpopulations. mRNA quantitation (y-axis) was obtained relative to the reference gene human acidic ribosomal protein P0 according to the method of Livak (Livak and Schmittgen, 2001). (*A*) p16 levels (*black bars*) were nearly undetectable in flow-sorted normal and psoriatic cells. (*B*) *In vitro,* p16 levels showed an increase in normal and psoriatic cells compared with freshly isolated cells; note the strong increase in the psoriatic bright cells. Results of sorted cells represent mean values ± SEM of three different patients, whereas cultured cells were based on four samples. NS, normal skin; PS, psoriatic skin.

Discussion

This study provides evidence that flow-sorted β_1-integrin dim (assumed transit amplifying) cells and β_1-integrin bright (putative stem) cells from normal and psoriatic skin are phenotypically and functionally different. From their growth behavior in culture, we conclude that psoriatic TAC are more advanced in their life cycle than the dim cells in normal skin. Furthermore, the strong downregulation of marker genes for regenerative maturation (psoriasis-related differentiation markers) in the cultured psoriatic dim cells in the absence of hyperproliferation suggests that proliferation and regenerative maturation/differentiation are coupled processes.

Since Jones and Watt demonstrated that high levels of the $\alpha_2\beta_1$ (a receptor for collagens and laminins), $\alpha_3\beta_1$ (a receptor for laminins and epiligrin), and $\alpha_5\beta_1$ integrin (a receptor for fibronectin) can distinguish SC from TAC and postmitotic differentiating cells in cultured human epidermis (Jones and Watt, 1993; Jones *et al*, 1995), β_1-integrin expression has been used to isolate β_1-integrin bright and β_1-integrin dim cells by fluorescence activating cell sorting.

This method allowed *in vitro* studies on fractions enriched for each cell type (Fig 1). But, high β_1-integrin expression marks approximately 25%–40% of the basal cells, whereas SC are estimated to comprise less than 10% of the basal cells (Jones *et al*, 1995). In psoriasis, β_1-integrin expression is not only confined to the basal layer but has also extended to the suprabasal layers (Hertle *et al*, 1992; Watt and Jones, 1993). In fact, it has even been suggested that suprabasal expression of integrins could be a pathogenic factor in psoriasis (Carroll *et al*, 1995). In this study, we therefore investigated the *in vivo* and *in vitro* characteristics of β_1-integrin bright and β_1-integrin dim keratinocytes derived from normal and psoriatic skin biopsies.

After inoculation of β_1-integrin bright and dim keratinocytes, the first colonies were observed around day 6 in cultures derived from normal skin and at approximately day 8 or 9 in cultures derived from psoriatic skin. In normal cultures, 60% confluence was reached after 15 d and 100% confluence at approximately 22 d. Remarkably, psoriatic cultures had only reached 60% confluence at 22 d after plating, suggesting a decreased proliferative activity compared with normal keratinocytes. In fact, the psoriatic β_1-integrin dim colonies did not reach confluence at all during the entire culture period of 30 d. Each well revealed only a few colonies, of which the majority was abortive, showing a keratinized centre (Fig 6). Subcloning studies of Barrandon and Green (1987) allowed classification of cultured keratinocyte clones into three different types. First, the holoclone was distinguished by a high reproductive capacity in the absence of terminal differentiation. Second, a paraclone consisted of cells that stopped dividing after a few rounds of mitosis and underwent terminal differentiation. And finally, meroclones were a mixture of cells of different growth potential, representing a transitional stage between a holoclone and a paraclone (Barrandon and Green, 1987). In our study, cultures from normal β_1-integrin bright and dim keratinocytes showed colonies resembling predominantly holoclones and meroclones, whereas the psoriatic keratinocytes, on the other hand, revealed colonies resembling holo- or meroclones in the β_1-integrin bright cultures, and predominantly paraclones in the β_1-integrin dim cultures (Fig 6). These findings suggest that psoriatic TAC are more advanced in their life cycle than the dim cells in normal skin, although both fractions were sorted alike on their low expression of the β_1-integrin.

Bata-Csorgo *et al* (1993) observed increased numbers and hyper-responsiveness of CD29 (β_1-integrin)$^+$ K1/10$^-$ cells in psoriatic compared with normal epidermis (Bata-Csorgo *et al*, 1993). These findings were attributed to SC hyperproliferation (Bata-Csorgo *et al*, 1995). It is also possible, however, that this abnormality is the result of an increased activity of "young" TAC, being cells that have just left the stem cell compartment. Furthermore, the relative proportions of CD29$^+$ K1/10$^-$ and CD29$^+$ K1/10$^+$ cells proved similar in normal and psoriatic epidermis (Bata-Csorgo *et al*, 1993), suggesting accelerated maturation of newly formed TAC, followed by loss of the CD29 label. Comparison of our results with these findings suggests that the SC hyperproliferation or the increased activity of young TAC in the psoriatic epidermis leads to an accelerated maturation of the latter cells, explaining the decrease of their proliferative activity *in vitro* compared with the dim cells in normal skin. In fact, in our previous work, an assessment of β_1-integrin bright and β_1-integrin dim cells derived from normal skin biopsies showed that the total number of colonies formed and the initial growth rate of the colonies were higher in the β_1-integrin dim cells than in the β_1-integrin bright subpopulation (van Rossum *et al*, 2004). These findings are more in line with the fact that TAC have a greater initial proliferative rate, at least in normal skin.

With respect to the mRNA levels of the β_1-integrin, no differences in the expression levels of β_1-integrin were found comparing the bright and the dim cells of normal skin, despite the protein differences. In the psoriatic dim cells, however, the mRNA levels were markedly downregulated. During culture, a small increase of the initial level was seen in the β_1-integrin bright cells of normal and psoriatic skin and in the β_1-integrin dim cells of normal skin, whereas the levels in the psoriatic β_1-integrin dim cells remained low (Fig 2*A, B*). Since downregulation of the β_1-integrin protein is associated with the onset of terminal differentiation (Adams and Watt, 1990; Hotchin and Watt, 1992; Hotchin *et al*, 1993, 1995; Watt and Hertle, 1994), the low mRNA levels detected in the psoriatic dim cells suggest a strong commitment to terminal differentiation. In fact, we found even lower mRNA levels in the psoriatic dim cells compared with the normal dim cells, again suggesting that the psoriatic TAC are ahead in their life cycle compared with their normal counterparts.

Expression of the β_1-integrin protein is not correlated with its functional state (Penas *et al*, 1998). In fact, in psoriasis, the suprabasal β_1-integrins were found to be in an inactive state, and the basal integrins mostly in a nonactive confirmation, suggesting that active β_1-integrins might be related to inhibition of proliferation, whereas inactive epitopes result in hyperproliferation *in vivo*. Indeed, when examining the levels of Ki-67 expression, we found virtually undetectable levels in normal keratinocytes, and 5-fold higher levels in psoriatic keratinocytes. *In vitro*, a strong upregulation of the Ki-67 level was seen in normal cells, whereas we noticed a strong downregulation in psoriatic keratinocytes and particularly in the psoriatic dim cells. This was confirmed by flow cytometric analysis of cultured keratinocytes, showing only 20% Ki-67-positive cells in the psoriatic dim fraction, whereas nearly all cells were Ki-67-positive in both normal fractions and approximately 50% in the psoriatic bright fraction. Similar results were found for the hyperproliferation-related keratin 6; as expected no expression was found in normal keratinocytes, but the psoriatic keratinocytes revealed a 3-fold higher mRNA level in psoriatic dim cells compared with bright cells. Cultured normal keratinocytes showed an upregulation, whereas psoriatic dim cells showed a 6-fold downregulation. Once more illustrating that although the psoriatic TAC are hyperproliferative *in vivo*, this phenotype ceases almost instantly *in vitro*, whereas the normal dim cells are still able to proliferate a number of times in culture.

The p63 transcription factor was suggested as a marker for SC, since it was abundantly expressed in holoclones but undetectable in paraclones (Pellegrini *et al*, 2001). In fact, mRNA levels showed highest expression in both the normal as the psoriatic bright cells, which is in keeping with this

study. In our culture system, however, p63 levels were strongly downregulated in all subpopulations (Fig 4*A*, *B*). Remarkably, freshly prepared normal and psoriatic keratinocytes revealed similar mRNA levels for collagen VII as seen for p63, again with a low expression *in vitro*. Flow cytometric analysis of β_1-integrin-positive keratinocytes displayed a small population of β_1-integrin bright and collagen VII bright cells with a low side-scatter phenotype. As these cells accounted for approximately 1%–2% of the cells measured, and both β_1-integrin and collagen VII are expressed in basal keratinocytes; obviously, these β_1-integrin bright/collagen VII bright cells necessitate further investigation and collagen VII might be a new potential SC marker.

Finally, we examined the β_1-integrin bright and β_1-integrin dim fractions of psoriatic skin for their expression levels of genes associated with regenerative maturation (Fig 5*A*, *B*). As expected, freshly prepared normal keratinocytes revealed undetectable mRNA levels of PA-FABP, psoriasin, and elafin. Psoriatic dim keratinocytes on the other hand, showed significantly higher mRNA levels of all three molecules compared with the levels detected in the psoriatic bright keratinocytes. Using 2D gel electrophoresis and northern blotting, a 2-fold upregulation of PA-FABP was found in the β_1-integrin dim population of cultured human foreskin keratinocytes (O'Shaughnessy *et al*, 2000), suggesting PA-FABP as a marker for TAC. In our hands, freshly prepared β_1-integrin dim keratinocytes derived from adult interfollicular epidermis displayed very low levels of this molecule, again suggesting that cultured foreskin keratinocytes differ from cultured adult keratinocytes. Although it was demonstrated that PA-FABP can form a complex with psoriasin (or S100A7), a member of the calcium binding S100 protein family (Hagens *et al*, 1999a, b), no upregulation of psoriasin was found in integrin dim cells (O'Shaughnessy *et al*, 2000). Our results, however, show a strong expression of psoriasin mRNA in psoriatic β_1-integrin dim keratinocytes. As psoriasin and PA-FABP were demonstrated to co-localize in differentiating keratinocytes of the psoriatic lesion (Hagens *et al*, 1999a), our findings clearly underline the strong commitment to differentiation in psoriatic β_1-integrin dim cells. Similar results were found regarding the expression levels of elafin. As expected, cultured normal keratinocytes showed a marked upregulation of their initial mRNA levels of all three molecules. Psoriatic dim cells *in vitro*, on the other hand, displayed a downregulation of the mRNA levels, suggesting a strong commitment toward differentiation with an unexpected loss of the psoriatic phenotype. These results suggest a remarkable different phenotype of the psoriatic TAC compared with its normal counterpart *in vivo* as well as *in vitro*. The defect noted *in vitro* was maintained for the duration of the culture experiment. The fact that the flow-sorted TAC-enriched fractions were not capable of inducing regenerative maturation anymore, despite their commitment to terminal differentiation, was unexpected. Perhaps, this is because of the absence of a hyperproliferative basal layer as shown by the low Ki-67 expression *in vitro* (Fig 3*B*), suggesting a loss of critical cell communication necessary for the psoriatic phenotype.

One can speculate as to the cause of the psoriatic TAC showing both loss of proliferative activity and regenerative phenotype once cultured. Our data suggest that TAC from psoriatic skin or the psoriatic β_1-integrin dim subpopulation are advanced in their life cycle compared with the β_1-integrin dim subpopulation derived from normal skin. We examined whether these subpopulations showed upregulation of proteins involved in negative cell cycle control, as for instance p16, p21, and p27. These cyclin-dependent kinase (Cdk) inhibitory proteins are believed to block transition from G_1 to the S phase of the cell cycle, and it is discussed whether upregulation of these proteins corresponds with a state of keratinocyte senescence (Chaturvedi *et al*, 1999; Sayama *et al*, 1999; Fu *et al*, 2003). More recently, the group of Nickoloff demonstrated that the tumor suppressor maspin is upregulated during keratinocyte senescence (Nickoloff *et al*, 2004), and we included this marker in our analysis. As expected, we found only low to undetectable expression levels in freshly sorted keratinocytes, but with the exception of p16, all expression levels remained low or unchanged after culturing. p16 showed upregulation in all normal and psoriatic subpopulations, with a marked increase in the psoriatic bright subpopulation (Fig 8*B*). Obviously, dim cells express higher levels of p16 when cultured, but the highest level of p16 expression was noted in the psoriatic bright subpopulation. Whether this correlates with keratinocyte aging or with keratinocyte senescence remains unclear, as evidence on p16 expression is conflicting. According to the work of Sayama, p16 appeared not to be associated with keratinocyte senescence (Sayama *et al*, 1999). In fact, the work of Fu demonstrated that although p16 showed upregulation in senescent keratinocytes cultured under serum-free conditions, the p16 expression level stayed low in keratinocytes cultured on feeders (Fu *et al*, 2003), suggesting that p16 expression is sensitive to culture conditions and probably not the ideal marker for senescence. Culture stress could be another factor, but this does not seem plausible as the expression of elafin, a keratinocyte marker induced by various forms of stress, showed an increase in normal keratinocytes, but it remained low in both the psoriatic bright and the dim cells (Fig 5*B*).

Obviously, there must be a tightly regulated balance between SC self-renewal and the generation of TAC that undergo a limited number of more rapid (amplifying) transit divisions before giving rise to postmitotic, terminally differentiating cells. It is generally believed that a subpopulation of SC resting in G_0 exists, which can enter the cell cycle when activated (Boezeman *et al*, 1987; Bickenbach and Dunnwald, 2000). It has always been assumed that the cells in the G_0 phase of the cell cycle were SC, but no direct evidence to prove this hypothesis has been published. Dunnwald *et al* (2003) have shown that mouse SC are cycling and not held out of the cell cycle as previously assumed. It is generally accepted that as soon as cells leave the SC compartment, they are committed to terminal differentiation, and this process is considered to be continuous and irreversible. In tape-stripped normal skin, however, the recruitment of quiescent cells originates in part from the layers above the basal cells, and in clinical clearance–relapse studies in psoriatic skin the expression of the Ki-67 antigen after relapse was even exclusively suprabasal (Rijzewijk *et al*, 1992; Castelijns *et al*, 1999). These studies suggest that there is an exclusive cell kinetic control point at

the level of the TAC. SC have an unlimited capacity for self-renewal and continuously generate TAC at a slow rate (long cell cycle time). On the other hand, the TAC in normal skin can either become quiescent (resting in G_0) at a specific restriction point, or proliferate rapidly for a limited number of cell divisions (short cell cycle time). In this way, a tissue can regulate cell production, and the production rate can be adapted on demand. The data presented in this study suggest an abnormality in the recruitment of cells from the transit amplifying specific restriction point in psoriatic epidermis, resulting in a different age distribution that is maintained *in vitro*. A better characterization of the epidermal germinative cell populations and the mechanisms involved in its regulation should lead to a better understanding of various hyperproliferative skin disorders such as psoriasis.

Materials and Methods

Biopsy procedure Normal epidermis was obtained from residual human skin from abdominoplasty in healthy individuals. Pieces of skin from about 4 cm^2 were taken from the residual skin and kept in MEM (Eagle, 25 mM Hepes with Earle's salts, without glutamine, Gibco BRL, Life Technologies, Grand Island, New York) with 1% vol/vol antibiotic–antimycotic solution (Gibco) for a maximum of 7 d at 4°C. Four millimeter punch biopsies were taken from this material. Additionally, 4 mm punch biopsies were obtained from psoriatic patients who did not receive topical treatment for at least 1 wk. In order to obtain sufficient cell numbers, each sample consisted of biopsies of four different patients. All psoriatic and surgical patients gave their written informed consent. Biopsies were kept in MEM with 10% vol/vol antibiotic–antimycotic solution for 2 h at 4°C before cell isolation procedures. The study was conducted according to the Declaration of Helsinki Principles. The medical ethical committee of the Radboud University Nijmegen approved all described studies.

Cell isolation and labeling procedure Biopsies were incubated overnight in PBS containing 2.5 mg per mL trypsin (trypsin 250, Difco Laboratories, Detroit, Michigan) at 4°C, as described previously (van Rossum *et al*, 2002). Ten percent fetal calf serum (Harlan Sera-Lab, Loughborough, UK) was added to stop trypzination and the epidermis and dermis were separated with pincers. The epidermal side of the dermis was gently scraped to ensure detachment of basal cells. Both the epidermis and dermis were mixed on a vortex to allow detachment of all keratinocytes. Subsequently, the remaining stratum corneum and dermis were removed and the cells were pelleted. The cells were resuspended and labeled with K20-fluorescein isothiocyanate (FITC) (DAKO A/S, Glostrup, Denmark), an antibody directed against β_1-integrin at a dilution of 1:20 for 45 min at 4°C. Next, the cell suspension was washed with 1% fetal calf serum/PBS and pelleted again. Finally, the cells were resuspended in medium and sorted.

Cell sorting The labeled cell suspensions were sorted on an EPICS Elite flow cytometer (Coulter, Luton, UK). A bandpass filter of 515–535 nm (green, FITC) was used to measure emitted light, while a gate in the right angle scatter (a measure of regularity) *versus* the forward scatter (a measure of size) was used to exclude debris. Gates in the mean FITC fluorescence *versus* the forward scatter were used to distinguish β_1-integrin-negative and β_1-integrin-positive cells. Of the β_1-integrin-positive population, next the 10% cells with the lowest mean FITC fluorescence were defined as β_1-integrin dim cells and the 10% cells with the highest mean FITC fluorescence were regarded as β_1-integrin bright cells (Fig 1). After sorting, cells were collected for either mRNA isolation of freshly sorted cells, or otherwise seeded for culture experiments. In each experiment, a six-well plate was filled with 20,000 cells per well, with either β_1-integrin dim cells, β_1-integrin bright cells, or as a control a subset of the β_1-integrin-positive cells.

Cell culture Sorted keratinocytes were seeded on lethally irradiated (3000 rad, 5 min) Swiss mouse 3T3 fibroblasts according to the Rheinwald–Green system (Rheinwald and Green, 1975). The cells were cultured in DMEM/F12 (3:1, vol/vol, Bio Whittaker, Walkersville, Maryland) containing 0.4 μg per mL hydrocortisone (Brunschwig Chemie BV, Amsterdam, the Netherlands), 10^{-6} M isoproterenol (Sigma, St Louis, Missouri), 100 i.u. per mL penicillin and 100 μg per mL streptomycin (Gibco), 6% fetal calf serum (Harlan Sera-Lab), and 10 ng per mL epidermal growth factor (Sigma). The six-well plates were kept in an incubator with 8% CO_2 in the air and at 37°C and 95% humidity.

Immunocytochemistry and flow cytometric analysis For immunocytochemical examination, parts of the sorted cells were cultured on tissue culture coverslips (Thermanox, ICN Biomedicals Inc., Aurora, Ohio). Approximately 7 d after seeding, colonies were visible and at an early (14 d) and late time point (21 d) coverslips were harvested for immunocytochemistry. Cells cultured on cover slips were fixed using 100% ice-cold acetone. Before usage, the coverslips were washed with excessive PBS. After blocking with PBS containing 50% normal rabbit serum, coverslips were incubated for 1 h with the following primary antibodies: anti-keratin 10 (RKSE60, Monosan, Uden, the Netherlands), anti-keratin 6 (LHK6B, Monosan) MIB-1 (Ki-67, DAKO A/S), anti-β_1-integrin (CD29; clone K20, DAKO A/S, and clone 4B4, Beckman Coulter BV, Mijdrecht, the Netherlands), anti-α_6-integrin (CD49f, Instruchemie, Delfzijl, the Netherlands), anti-collagen VII (Clone LH7.2, Novocastra Laboratories, New Castle upon Tyne, UK), anti-transferrin receptor (CD71, Immunotech, Marseille, France), and anti-p63 (clone 7JUL, Novocastra Laboratories). After a washing step, coverslips were incubated for 20 min with rabbit anti-mouse immunoglobulins (DAKO A/S) and subsequently with mouse PAP (DAKO A/S). After a washing step, coverslips were incubated by diaminobenzidine (DAB, liquid DAB+ large volume substrate-chromogen solution, DAKO A/S) for 2 min and incubating the coverslips in demineralized water terminated the reaction. Finally, coverslips were mounted in glycerol gelatin (Sigma) without counter-staining.

In order to obtain quantitative data, we performed flow cytometric measurements of the markers mentioned previously. For this reason, another fraction of the sorted cells was cultured in six-well plates as described above and harvested at approximately 60% and 100% confluency. Cells were trypsinized, and subsequently one fraction was fixed in 70% ice-cold ethanol and stored at −20°C until staining procedures, whereas another portion was measured unfixed. Antibodies were directly labeled or otherwise labeled using a Zenon One labeling kit containing R-phycoerythrin (orange, R-PE) or Alexa Fluor 488 (green, Molecular Probes, Eugene, Oregon). Antibodies directed against keratin 10 (RKSE60), keratin 6 (LHK6B), and p63 (7JUL) were obtained from Monosan. Antibodies against Ki-67 (Ki-67-RPE) and β_1-integrin (CD29-FITC; clone K20) were purchased from DAKO A/S. Antibodies against β_1-integrin (clone 4B4-PE, Beckman Coulter BV), α_6-integrin (CD49f-FITC, Instruchemie), β_4-integrin (CD104-FITC, Kordia Life Sciences BV, Leiden, the Netherlands), collagen VII (LH7.2, Novocastra Laboratories), and transferrin receptor (CD71-PE, Immunotech) were obtained as listed.

Real-time quantitative PCR Extraction of mRNA of both freshly sorted and cultured human keratinocytes, followed by the generation of first-strand cDNA, was performed as described previously (Zeeuwen *et al*, 2001). The reverse transcriptase reaction products were used for quantitative real-time PCR amplification, which was performed with the MyiQ Single-Color Real-Time Detection System for quantification with Sybr Green and melting curve analysis (Biorad, Richmond, California). Primer sequences for α_6-integrin, β_1-integrin, β_4-integrin, CD71, collagen VII, keratin 10, keratin 6,

Table I. Primer pairs used for quantitative real-time PCR

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	E	R^2
α_6-integrin	tgcgagccttcattgatgtg	cgaacctgagtgcctgcat	1.98	0.948
β_1-integrin	agttgcagtttgtggatcactgat	aaagtgaaacccggcatctg	2.16	0.998
β_4-integrin	tggcaaagcagccgatct	tgttactggttacttggcctcatg	2.11	0.989
CD71	tgaaaaggtcatagctgagattcct	acatgccacatgctttcatttaa	1.91	0.979
Collagen VII	ggccagttcatcgcatctg	gctcttcctcctctgcatgaga	1.93	0.991
hARP	caccattgaaatcctgagtgatgt	tgaccagcccaaaggagaag	2.00	0.997
Keratin 6	agagaatgaatttgtgactctgaagaag	tacaaggctctcaggaagttgatct	2.07	0.999
Keratin 10	tggttcaatgaaaagagcaagga	gggattgtttcaaggccagtt	1.95	0.998
Ki-67	aaaccaacaaagaggaacacaaatt	gtctggagcgcagggatattc	2.15	0.991
p63	caaagaacggtgatggtacgaa	cacggcccctcactggta	2.17	0.966
PA-FABP	accctgggagagaagtttgaaga	tgtaaagttgcagacagtctgagtttt	2.18	0.994
Psoriasin	cttccttagtgcctgtgacaaaaa	aaggacagaaactcagaaaaatcaatct	1.92	0.998
Elafin	catgagggccagcagctt	tttaacaggaactcccgtgaca	2.00	0.999
p16	cggaaggtccctcagacatc	tctaagtttcccgaggtttctca	2.13	0.999
p21	gcagaccagcatgacagatttct	ggattagggcttcctcttgga	1.99	0.999
p27	gtcaaacgtaaacagctcgaattaag	ttcatgtatatcttccttgcttcatca	1.88	0.999
Maspin	gaatgtcctttcagaatcagcaaga	tggcctcaagattcatcatttg	2.21	0.974

E is efficiency as fold increase in fluorescence per PCR cycle and R^2 is the correlation coefficient for the PCR dilution series.
PA-FABP, psoriasis-associated fatty acid binding protein; hARP, human acidic ribosomal protein P0.

Ki-67, p63, PA-FABP, psoriasin, SKALP/elafin, p16, p21, p27, maspin, and the housekeeping gene human acidic ribosomal protein P0 (hARP) are depicted in Table I. All primers were obtained from Biolegio (Biolegio BV, Malden, the Netherlands). DNA was PCR-amplified under the following conditions: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, with data collection in the last 30 s. For all PCRs, iQ SYBR Green Supermix (Biorad) was used in the reaction. All primer concentrations were 300 nM in a total reaction volume of 25 μL. PCR reactions were performed in duplicate. The amount of each mRNA in both keratinocyte subpopulations was normalized to the amount of hARP in the same sample. Relative mRNA expression levels of all examined genes were measured using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

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