Long-term expansion of human functional epidermal precursor cells: promotion of extensive amplification by low TGF-β1 concentrations

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Summary

We have previously introduced the concept of high proliferative potential-quiescent (HPP-Q) cells to refer to primitive human hematopoietic progenitors, on which transforming growth factor-β1 (TGF-β1) exerts a pleiotropic effect. TGF-β1 confers to these slow-dividing cells a mitogenic receptor^{low} phenotype and maintains immature properties by preventing differentiation and apoptosis. However, the effect of TGF-β1 on long-term expansion has not yet been clearly demonstrated. Here, we describe the characterization of a human skin keratinocyte subpopulation, highly enriched for primitive epidermal precursors, on the basis of high adhesion capacity (Adh^{+++}) and low expression of the epidermal growth factor receptor (Adh^{+++}EGF-R^{low}). In our standard culture condition without feeder cells, the mean estimated output for cells from an unfractionated population of primary foreskin keratinocytes was 10^7-10^8, increasing to 10^{12}-10^{13} in cultures initiated with selected Adh^{+++}EGF-R^{low} precursors. Characterization of these cells revealed a hitherto unknown property of TGF-β1: its addition at a very low concentration (10 pg/ml) in long-term cultures induces a very significant additional increase of expansion. In this optimized system, outputs obtained in cultures initiated with Adh^{+++}EGF-R^{low} cells repeatedly reached 10^{16}-10^{17} (~60 population doublings, ~4×10^{18} keratinocytes produced per clonogenic cell present in the initial population). At the molecular level, this effect is associated with an increase in Smad1, Smad2 and Smad3 phosphorylation and an increase in α6 and β1 integrin expression. No such effect could be observed on mature keratinocytes with low adhesion capacity (Adh$^{-/+}$). We finally demonstrated that the progeny of Adh^{+++}EGF-R^{low} precursors after long-term expansion is still capable of generating a pluristratified epidermis in a model for skin reconstruction. In conclusion, after further characterizing the phenotype of primitive epidermal precursors, we demonstrated a new function of TGF-β1, which is to promote undifferentiated keratinocyte amplification.

Key words: Human epidermal precursor, Expansion, Feeder layer-free culture, TGF-β1, High proliferative potential, Reconstructed epidermis, EGF-R

Introduction

Two major obstacles to the study of adult somatic stem cells are the paucity of specific selection markers and our current inability to understand fully the controls of stemness and to exploit the capacity for stem cell self-renewal. In two previous issues of *J. Cell Sci.* (Fortunel et al., 1998; Batard et al., 2000), we have introduced the working model of high proliferative potential-quiescent (HPP-Q) cells to refer to primitive human hematopoietic progenitor cells, on which transforming growth factor-β1 (TGF-β1) exerts an important regulatory role. According to this model, (1) TGF-β1 maintains these cells in a quiescent or slow-cycling state, in part by downmodulating various cytokine receptors, resulting in a mitogenic receptor^{low} phenotype, and thus providing a tool to select this subpopulation (Fortunel et al., 1998); and (2) TGF-β1 may also participate in the control of hematopoietic stem/progenitor cell immaturity. This second function is suggested by the fact that TGF-β1 maintains a high level of the cell-surface expression of hematopoietic cell immaturity markers, such as CD34, throughout successive divisions (Batard et al., 2000). However, because hematopoietic stem and progenitor cells spontaneously differentiate into several lineages and do not remain as a homogenous population in culture, it appeared complex to study the effect of TGF-β1 on long-term self-renewal in this system. As presented in this report, we found that the human epidermal precursor cell compartment represents a unique model to analyze the effect of TGF-β1 on long-term self-renewal of functional undifferentiated somatic cells.

Most studies performed to identify cell-surface markers expressed by primitive keratinocytes have focused on molecules involved in cell adhesion. It has been reported that early keratinocytes of the basal layer of the epidermis express the α2β1 and α3β1 integrins (Peltonen et al., 1989; Carter et

al., 1990). A high expression level of the β1 integrin chain (CD29) has been associated with the high plating efficiency of primitive human keratinocytes in culture (Jones and Watt, 1993; Jones et al., 1995), and with epidermal stem cell functional properties in a murine xenograft model (Jones et al., 1995). More recently, keratinocytes with the greatest proliferative capacity have been shown to express a high level of the α6 integrin chain (CD49f) and, by contrast, a low to undetectable level of the transferrin receptor (CD71) (Li et al., 1998).

In this study, we have first investigated the possibility of isolating a cell subpopulation that was enriched for primitive epidermal precursors with high proliferative potential and maintaining a durable capacity to generate a pluristratified epidermis throughout expansion. This was achieved by selecting cells presenting a mitogenic receptor^{low} cell-surface phenotype, as we previously described for primitive hematopoietic cells in the HPP-Q working model. This selection has been performed on the basis of the cell-surface expression level of the epidermal growth factor receptor (EGF-R), which has largely been described to exert a mitogenic effect on keratinocytes (Cook et al., 1991). On the basis of our data suggesting that TGF-β1 could participate in the control of hematopoietic progenitor cell immaturity, we then explored the capacity of TGF-β1 to control the cell cycling and long-term expansion of immature keratinocytes. The effect of extremely low, yet physiological, concentrations of TGF-β1 (10-30 pg/ml) was analyzed, revealing a hitherto undescribed property of TGF-β1. At these low concentrations, TGF-β1 efficiently promotes the long-term expansion of undifferentiated epidermal precursor cells in a feeder layer-free culture condition.

Materials and Methods

Isolation of primary basal keratinocytes

Human neonatal foreskins obtained at circumcision provided the input material. Samples were first treated with gentamicin (Invitrogen, Paisley, UK). Epithelial sheets were separated from the derma after an overnight incubation with dispase (Boehringer, Mannheim, Germany) at 4°C, followed by 45 minute incubation at 37°C. Basal keratinocytes were isolated by trypsinization (Boehringer) for 15 minutes and fractionated into Adh$^{-/+}$ and Adh^{+++} populations. Samples were deposited on a plastic surface coated with type I collagen (Sigma) and incubated for 12 minutes at 37°C. The Adh$^{-/+}$ cell population were keratinocytes with low adhesion capacity that did not attach to type I collagen within 12 minutes, and the Adh^{+++} population were keratinocytes with high adhesion capacity that remained attached to the substrate after washing. A 12 minute adhesion period was sufficient to obtain a significant enrichment in clonogenic keratinocytes.

Immunofluorescence staining and cell sorting by flow cytometry

For the detection of cell-surface EGF-R, keratinocytes were first incubated for 15 minutes with rat γ-globulins (Jackson ImmunoResearch Laboratories, West Grove, PA), and then for 30 minutes with a non-conjugated monoclonal anti-human EGF-R mouse IgG_{2b} (EGFR1 clone; Dako, Glostrup, Denmark) or an isotypic control from the same species (mouse IgG_{2b}) (Immunotech, Marseille, France). Samples were washed twice and then incubated for 30 minutes with a rat anti-mouse IgG_{2a+b}-PE antibody (Becton Dickinson, San Jose, CA). Analyses and cell sorting were performed using a Vantage Fluorescence Activated Cell Sorter (FACS) (Becton Dickinson).

Cell-cycle and immuno-phenotypic analyses by laser scanning cytometry

For cell-cycle analysis, keratinocytes were plated on glass slides and grown without exogenous TGF-β1 until formation of multicellular clones. TGF-β1 (R&D Systems, Abingdon, UK) was then added to the medium at concentrations ranging from 10 to 3000 pg/ml. Samples were processed 24 hours later for cell-cycle analyses. Cells were fixed and permeabilized in acetone for 30 minutes at –20°C. They were then treated with DNase-free RNase A (Boehringer) for 20 minutes at 37°C, and incubated for at least 15 minutes at room temperature with 20 mg/ml propidium iodide (Sigma). The distribution of the keratinocytes into the G_0/G_1 and $S+G_2/M$ phases of the cell cycle was analyzed in each condition using a Laser Scanning Cytometer (LSC) (CompuCyte, Cambridge, MA). Acquisition and data analysis were performed using Wincyte software (CompuCyte). For the analysis of α6 and β1 integrin expression, keratinocytes from cultures initiated with Adh^{+++}EGF-R$^{-/+}$ cells, previously cultured with or without 10 pg/ml TGF-β1 during four successive passages, were plated on glass slides and grown for three additional days in the same culture conditions. Cells were then fixed in methanol for 5 minutes at –20°C. Prior to α6 or β1 integrin immunostaining, cells were washed in PBS containing 0.2% BSA (PBS/BSA), then incubated respectively with rat or mouse γ-globulins (Jackson ImmunoResearch Laboratories) for 15 minutes, and then with a FITC-conjugated monoclonal anti-human integrin α6 chain (CD49f) rat IgG_{2a} (GoH3 clone; Pharmingen, San Diego, CA), or a FITC-conjugated monoclonal anti-human integrin β1 chain (CD29) mouse IgG_{2a} (K20 clone; Immunotech) for 30 minutes. For Smad phosphorylation studies, keratinocytes from cultures initiated with Adh^{+++}EGF-R$^{-/+}$ cells were plated on glass slides and grown for 72 hours with or without 10 pg/ml TGF-β1. After fixation in methanol for 5 minutes at –20°C, samples were washed twice in PBS/BSA, and then successively incubated for 15 minutes with irrelevant chicken Ig (Jackson ImmunoResearch Laboratories), for 30 minutes with non-conjugated polyclonal rabbit anti-human phosphorylated (p)-Smad1 (Ser463/Ser465) antibodies (sc-12353-R; Santa Cruz Biotechnology, Santa Cruz, CA) or non-conjugated polyclonal rabbit anti-human p-Smad2 and 3 (Ser433/Ser435) antibodies (sc-11769-R; Santa Cruz Biotechnology), and then for 30 minutes with Alexa Fluor488-conjugated chicken anti-rabbit IgG (Molecular Probes, Eugene, OR). Appropriate negative controls were used to determine background signals. The percentage of positive cells, as well as the median value of fluorescence (arbitrary units, a.u.) measured at the level of the population, were evaluated in each culture condition by LSC.

Long-term expansion assays

Cultures were carried out on plastic substrates (25 cm^2 Falcon) in a serum-free medium containing 100 pg/ml EGF, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.4% (v/v) bovine pituitary extract (KGM Bullet Kit; BioWhittaker, Clonetics, San Diego, CA). Cultures were initiated with selected populations of keratinocytes plated at 2400 cells/cm^2. After reaching no more than 70-80% confluence, expanded keratinocytes were detached by trypsinization (Boehringer), counted and replated at 2400 cells/cm^2. Cultures were continually passaged until the growth capacity of the cells was exhausted. Culture medium supplemented or not with the specified concentrations of recombinant human TGF-β1 (R&D Systems) was completely renewed three times per week. The cumulated total cell outputs were calculated assuming that all the cells from the previous passage had been replated. For each cell population and growth factor condition studied, cultures were performed in quadruplicate. At each step, cell viability was evaluated

by trypan blue exclusion. Statistical analyses were performed using the Student's *t* test.

Reconstructed epidermis

Substrate

De-epidermized human dermis (DED) was prepared according to the technique described by Régnier et al. (Régnier et al., 1981). Briefly, split-thickness human skin, obtained at plastic surgery, was floated in magnesium/calcium-free phosphate-buffered saline at 37°C for 10 days. Thereafter, the epidermis was separated from the dermis. Dermal cells were killed by serial freezing and thawing, and the cell-free dermis was stored at –20°C until use.

Reconstruction of an epidermis

Human foreskin keratinocytes cultured as described previously were seeded at different passages onto the DED and cultured for 6 days in Dulbecco's modified Eagle medium/Ham F12 (Invitrogen), containing 10% fetal calf serum (Invitrogen), 10 ng/ml EGF (BD Biosciences, USA), 0.4 μg/ml hydrocortisone (Sigma), 10^{-6} M isoproterenol (Sigma), 5 μg/ml transferrin (Sigma), 2×10^{-9} M triiodothyronine (Sigma), 1.8×10^{-4} M adenine (Sigma) and 5 μg/ml insulin (Sigma). Thereafter, the cultures were raised to the air-liquid interface, and were continued in the absence of isoproterenol, transferrin, triiothyronine and adenine. Histological examination of the reconstructed epidermis was performed after 7 days of culture.

Results and Discussion

Using a mitogenic receptor^{low} cell-surface phenotype, as described for primitive hematopoietic cells (Sansilvestri et al., 1995; Fortunel et al., 1998; Fortunel et al., 2000c), we demonstrated that primitive and highly proliferative human epidermal precursor cells can be isolated. Selection of these epidermal precursor cells was achieved by two successive enrichement steps. Then, through further functional characterization of this human epidermal precursor cell subpopulation, we found that a low physiological concentration of TGF-β1 is able to promote long-term expansion of undifferentiated keratinocytes in a feeder layer-free culture system.

First enrichment step: selection of keratinocytes with high adhesion capacity (Adh^{+++})

An initial enrichment step was based on the knowledge that maturing basal keratinocytes lose their capacity to adhere to extracellular matrix components (Adams and Watt, 1990). The basis of this method is provided by the work of Jones and Watt, who demonstrated that the most primitive keratinocytes with characteristics of stem cells (high colony-forming efficiency and long-term proliferative potentiel) adhered most rapidly to type IV collagen, whereas later keratinocyte populations (transit amplifying cells and post-mitotic differentiated keratinocytes) adhered more slowly (Jones and Watt, 1993). Cells with the highest adhesion capacity (Adh^{+++}) were selected here on a substrate coated with type I collagen, thus eliminating most of the post-mitotic mature keratinocytes. Morphological observation of the two cell populations obtained after adhesion-based separation indicated that the selected Adh^{+++} population is homogenously composed of small-sized undifferentiated cells of less than 12 μm in diameter, whereas the non-selected Adh$^{-/+}$ population is largely heterogenous, mainly composed of differentiating keratinocytes increasing in size. The Adh^{+++} population represented only 10.4% of the total keratinocytes obtained from neonatal foreskins, but was significantly enriched with clonogenic cells that effectively contribute to culture initiation. In a short-term assay, 2.3% of selected Adh^{+++} keratinocytes possessed a clone-forming ability, whereas only 0.2% of the Adh$^{-/+}$ keratinocytes were clonogenic (n>10 independent samples). This is in agreement with a previous study showing an inverse correlation between the size of keratinocytes and their clonogenic potential (Barrandon et al., 1985).

Comparison of the long-term proliferative potential of these populations confirmed that Adh^{+++} keratinocytes were more primitive than Adh$^{-/+}$ keratinocytes (Fig. 1A). The estimated output for one plated Adh^{+++} keratinocyte was at least 100-fold higher than that of unfractionated keratinocytes. By contrast, Adh$^{-/+}$ keratinocytes expressed only a limited proliferative potential, giving rise to ~500-fold fewer cells than a similar number of unfractionated keratinocytes. In the typical experiment shown in Fig. 1A, mean estimated outputs from one plated keratinocyte were 10^9-10^{10} for Adh^{+++} cells, 10^6-10^7 for unfractionated cells, and 10^3-10^4 for Adh$^{-/+}$ cells, corresponding respectively to 31-32 population doublings (PDs), 23-24 PDs, and 12-13 PDs (unfractionated versus Adh^{+++} or versus Adh$^{-/+}$ cells; P<0.01, n>5 experiments). It has been reported that, in post-confluent sheets of cultured human keratinocytes, a process of autoregulation adjusts the frequency of primitive cells independently of their initial frequency in the culture (Jones et al., 1995). The results presented in Fig. 1A show that an initial enrichment in primitive cells at the onset of culture results in an increased cumulated expansion throughout several successive passages, which on the contrary suggests no significant autoregulation of primitive cell frequency. This difference might be explained by the fact that, in the long-term culture experiments presented here, cells were systematically passaged before reaching confluence in order to limit, as far as possible, any regulation of cell fate linked to contact inhibition and homeostasis.

Second enrichment step: sorting of Adh^{+++} epidermal precursor cells presenting a low level of EGF-R expression (Adh^{+++}EGF-R^{low})

In a second enrichment step, we separated by FACS the Adh^{+++} population into four subpopulations of equal size, with increasing levels of cell-surface expression of EGF-R from Adh^{+++}EGF-R$^{-/+}$ to Adh^{+++}EGF-R^{++++} (sorting gates are shown in Fig. 1B). Keratinocytes with the greatest proliferative potential were mostly included in the Adh^{+++}EGF-R$^{-/+}$ subpopulation (~20% of Adh^{+++} cells, ~2% of total keratinocytes). Adh^{+++}EGF-R$^{-/+}$ (Adh^{+++}EGF-R^{low}) cells provided the highest cumulative expansion in long-term cultures compared with Adh^{+++}EGF-R^{++++} (Adh^{+++}EGF-R^{high}) cells (P<0.01, n=5 experiments). In the representative experiment shown in Fig. 1C, the mean output from one plated Adh^{+++}EGF-R$^{-/+}$ keratinocyte was 10^{12}-10^{13} (41-42 PDs, 13 successive passages). Adh^{+++}EGF-R^{++} and Adh^{+++}EGF-R^{+++} keratinocytes promoted a less-efficient

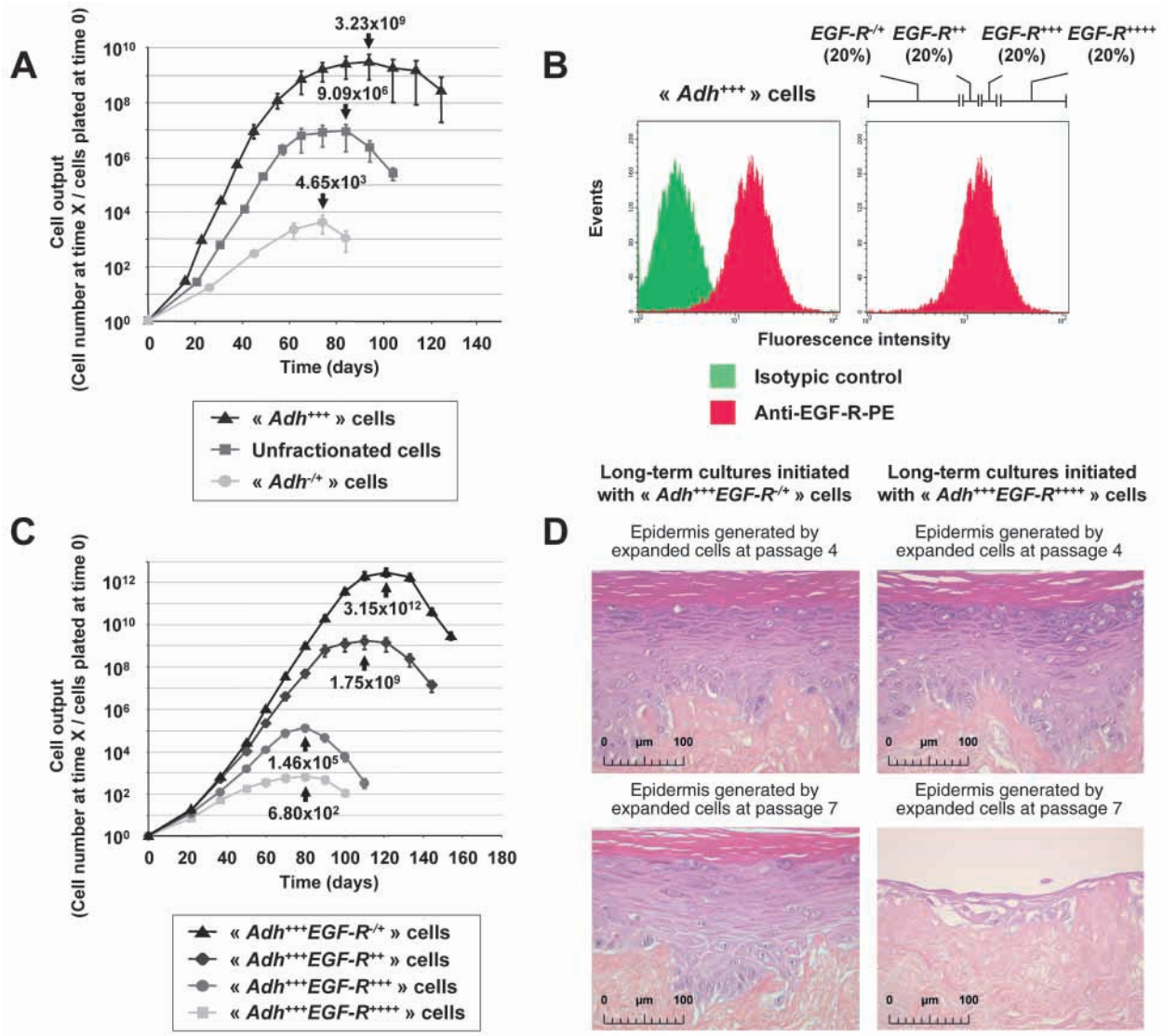

Fig. 1. Characterization of the Adh^{+++}EGF-R$^{-/+}$ epidermal stem cell subpopulation. (A) Basal keratinocytes were separated on the basis of their adhesion properties. The cell population designated as Adh$^{-/+}$ is composed of keratinocytes with low adhesion capacity, and the Adh^{+++} population is composed of keratinocytes with high adhesion capacity. Cells of each population were studied for their long-term expansion potential. Expansion curves are expressed as a cumulated cell output. Data represent means±s.d. of four replicate cultures from one typical experiment. (B) Cells of the Adh^{+++} population were labeled to analyze their level of EGF-R cell-surface expression by flow cytometry. Sorting gates were defined to isolate four subpopulations: the Adh^{+++}EGF-R$^{-/+}$ subpopulation contains the 20% of the Adh^{+++} keratinocytes presenting the lowest level of EGF-R expression; the Adh^{+++}EGF-R^{++++} subpopulation contains the 20% of the Adh^{+++} keratinocytes presenting the highest level of EGF-R expression; the Adh^{+++}EGF-R^{++} and Adh^{+++}EGF-R^{+++} subpopulations each contained the 20% of the Adh^{+++} keratinocytes presenting intermediate levels of EGF-R expression. (C) The long-term proliferative potential of Adh^{+++}EGF-R$^{-/+}$, Adh^{+++}EGF-R^{++}, Adh^{+++}EGF-R^{+++} and Adh^{+++}EGF-R^{++++} keratinocytes were compared. Data represent means±s.d. of four replicate cultures from one typical experiment. (D) Capacity of cell subpopulations, sorted according to the level of cell-surface EGF-R expression, to generate a reconstructed epidermis. Selected keratinocytes of the Adh^{+++}EGF-R$^{-/+}$ and Adh^{+++}EGF-R^{++++} subpopulations were expanded in defined culture conditions, and then seeded on to a dermal substrate at an early passage (p4) and a late passage (p7) to evaluate their capacity to produce a pluristratified epidermis. Histological preparations shown are from one typical experiment.

expansion: cell outputs of 10^9-10^{10} and 10^5-10^6 (respectively 30-31 and 17-18 PDs, 12 and 9 successive passages). The Adh^{+++}EGF-R^{++++} subpopulation showed the lowest proliferative capacity with a cell output of 10^2-10^3 (9-10 PDs, 8 successive passages).

Cell sorting based on the EGF-R^{low} phenotype, as in the HPP-Q working model, is shown here to be effective for selection, within the Adh^{+++} population, of the primitive keratinocytes possessing the greatest expansion potential in long-term culture. It is important to note that EGF-R is not a specific marker of the epidermal stem cell compartment. This tyrosine kinase receptor is widely expressed in the basal and suprabasal layers of the epidermis, regulating not only primitive epidermal cell cycling, but also commitment and terminal differentiation of later keratinocytes (Peus et al., 1998). The starting population used here to sort EGF-R^{low}

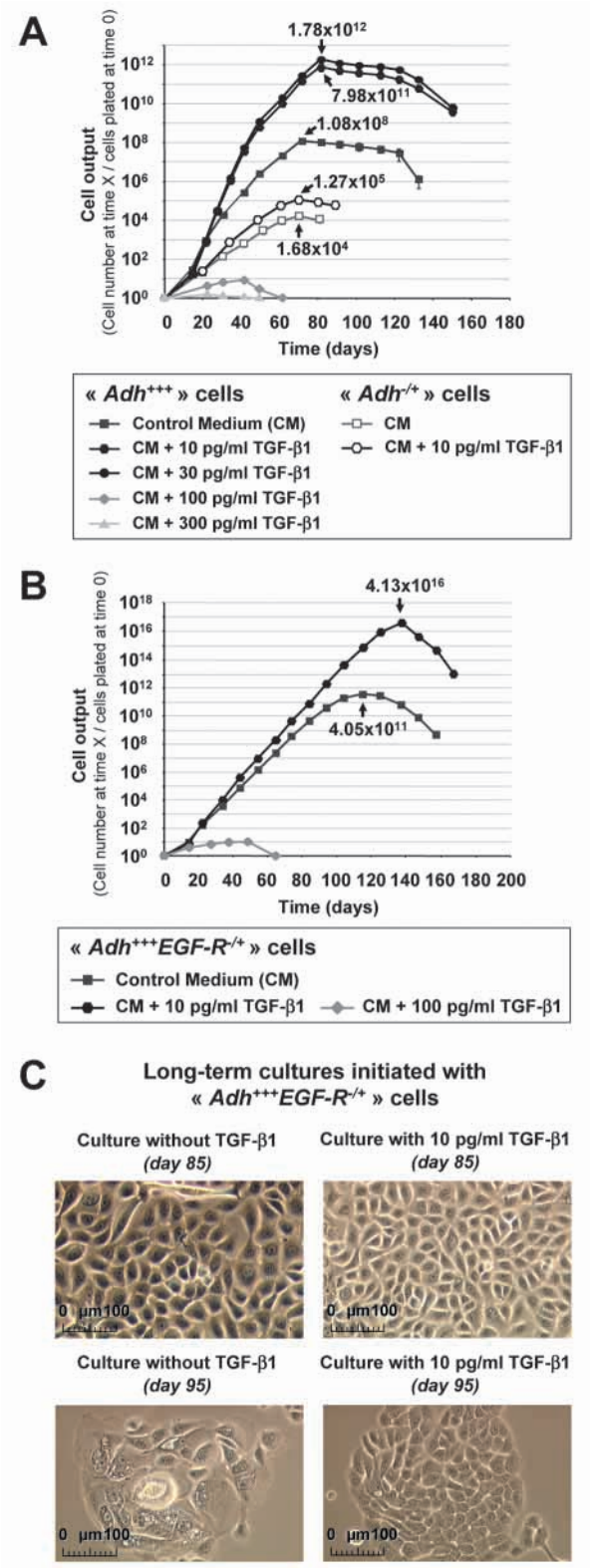

Fig. 2. Dose-response effect of TGF-β1 on the proliferation and expansion of keratinocytes. (A) Long-term cultures were initiated with keratinocytes of the Adh^{+++} or $Adh^{-/+}$ populations, and maintained with or without addition of exogenous TGF-β1 at a concentration of 10, 30, 100 or 300 pg/ml throughout the culture period, as specified. Results are expressed as a cumulated cell output. (B) Long-term cultures were initiated with sorted keratinocytes of the $Adh^{+++}EGF\text{-}R^{-/+}$ subpopulation, and maintained in the presence or absence of exogenous TGF-β1 at a concentration of 10 or 100 pg/ml throughout the culture period. Results are expressed as a cumulated cell output. Data represent means±s.d. of four replicate cultures from one typical experiment. (C) Long-term cultures were initiated with sorted keratinocytes of the $Adh^{+++}EGF\text{-}R^{-/+}$ subpopulation and maintained in the presence or absence of TGF-β1 at the concentration that promotes self-renewal of immature keratinocytes (10 pg/ml). Typical morphology of the cells obtained in these two conditions after 85 and 95 days of culture is illustrated in Fig. 2C.

epidermal precursors consisted of Adh^{+++} cells and not of unfractionated keratinocytes. Indeed, it appeared more appropriate to work on a cell population depleted in more mature keratinocyte populations, in which the level of EGF-R cell-surface expression is not related to stem cell cycling.

Capacity of primitive $Adh^{+++}EGF\text{-}R^{low}$ epidermal precursors to generate a pluristratified epidermis

Another feature of $Adh^{+++}EGF\text{-}R^{-/+}$ ($Adh^{+++}EGF\text{-}R^{low}$) keratinocytes that was evaluated was their organogenic potential. Subpopulations of keratinocytes ($Adh^{+++}EGF\text{-}R^{high}$ or $Adh^{+++}EGF\text{-}R^{low}$) were passaged up to seven times and then seeded onto a dermal substrate. At early passages (p4), keratinocytes initially expressing either a high or a low level of EGF-R were able to form an epidermis (Fig. 1D). In both cases, the characteristic epidermal differentiation pattern was observed: (1) a basal layer containing polygonal cells oriented perpendicular to the underlying dermis; (2) 3-4 layers of spinous cells; (3) 4-6 layers of granular cells characterized by the presence of keratohyalin granules; and (4) anucleated, flattened cornified cells forming a compact stratum corneum. At later passages (p7), only keratinocytes displaying the highest proliferative capacity, namely the $Adh^{+++}EGF\text{-}R^{low}$ subpopulation, were still able to form an epidermis (Fig. 1D), demonstrating a greater capacity of this subpopulation to maintain their organogenic potential throughout in vitro expansion.

Promotion of epidermal precursor cell amplification by low TGF-β1 concentrations

The cytokine TGF-β1 has been described to have a pleiotropic effect on hematopoietic $CD34^+$ stem and progenitor cells by either maintaining them in quiescence (Fortunel et al., 1998; Hatzfeld et al., 1991; Fortunel et al., 2000a; Fortunel et al., 2000b), or by preventing differentiation and apoptosis (Batard et al., 2000; Pierelli et al., 2000). However, the effect of TGF-β1 on long-term expansion of progenitor cells has not yet been demonstrated in a mammalian system. The isolation and characterization of the $Adh^{+++}EGF\text{-}R^{low}$ keratinocyte epidermal precursors revealed a novel function of TGF-β1. It appears to act as a long-term expansion-promoting factor for human primitive keratinocytes. Whereas the concentrations previously used are 10-1000-fold higher, our results demonstrated that, when present at extremely low but physiological concentrations (10-30 pg/ml), TGF-β1 efficiently increased primitive keratinocyte expansion.

In long-term cultures initiated with Adh^{+++} keratinocytes (Fig. 2A shows one typical experiment), the mean estimated

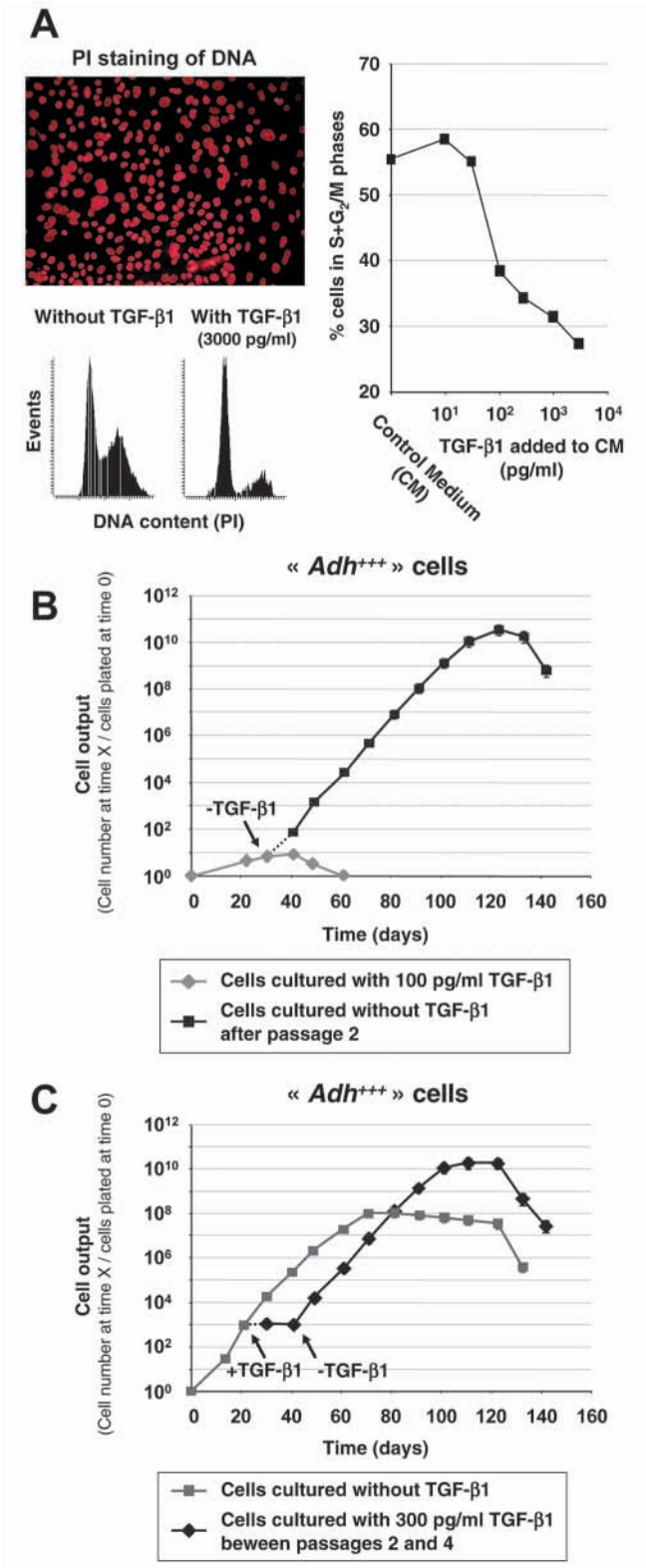

Fig. 3. Reversibility of the inhibitory effect of high TGF-β1 concentrations on keratinocyte cell cycling. (A) Cells of the Adh^{+++} population initially grown on slides without TGF-β1 were cultured for 24 hours with this factor added to the medium at a concentration of 0, 10, 30, 100, 300, 1000 or 3000 pg/ml. Samples were then processed for cell-cycle analysis by Laser Scanning Cytometry (LSC). Data from one typical experiment are shown (image of fluorescent nuclear DNA staining and cell-cycle analyses). (B) Long-term cultures were initiated with keratinocytes of the Adh^{+++} population and maintained in the presence of 100 pg/ml exogenous TGF-β1 up to day 30. Half of the cultures were continued from day 30 without addition of TGF-β1 and the rest were continued in the presence of 100 pg/ml exogenous TGF-β1. (C) Long-term cultures were initiated with keratinocytes of the Adh^{+++} population and maintained up to day 21 without addition of TGF-β1. Half of the cultures were continued from day 21 to day 41 in the presence of 300 pg/ml exogenous TGF-β1, and then from day 41 without addition of TGF-β1. The other half were continued from day 21 without addition of TGF-β1. Data represent means±s.d. of four replicate cultures from a typical experiment.

output for a single plated cell was 10^8-10^9 in the control condition, and 10^{11}-10^{12} when 10 pg/ml TGF-β1 was added to the medium. Calculations made to cumulate the results from independent cultures indicated that this represents a mean increase from 28-29 to 37-38 PDs (P<0.01, n=5 experiments). By contrast, 10 pg/ml TGF-β1 did not increase amplification of $Adh^{-/+}$ keratinocytes more than tenfold (from ~10^4 to ~10^5; Fig. 2A), which is about 1000-fold less than the effect observed on Adh^{+++} cells (from ~10^8 to ~10^{12}; Fig. 2A). These results suggest that this effect of TGF-β1 concerns mainly the most primitive cells. The slight increase in $Adh^{-/+}$ keratinocyte expansion might be due to the fact that the adhesion-based enrichment procedure was not effective in removing 100% of the Adh^{+++} cells from the $Adh^{-/+}$ population.

An inhibition of keratinocyte proliferation by TGF-β1 was observed in our culture system when high, but nevertheless physiological, concentrations (=100 pg/ml) were used (Fig. 2A,B). A significant reduction of the percentage of keratinocytes in $S+G_2/M$ phase of the cell cycle was observed 24 hours after addition of TGF-β1 at concentrations of ⩾100 pg/ml (Fig. 3A). It is important to note that this growth-inhibitory effect appeared to be reversible. Indeed, keratinocytes initially cultured for 30 days with an inhibiting concentration of TGF-β1 started to divide as soon as the addition of the factor was stopped, and subsequently they did not show any reduced capacity to proliferate and expand (Fig. 3B). Moreover, the proliferation of keratinocytes initially cultured for 21 days without exogenous TGF-β1 could be transiently inhibited by TGF-β1 (⩾100 pg/ml) for 20 days, without any alteration of their subsequent long-term expansion potential (Fig. 3C). These observations confirm those of a previous study, showing that exposure for 48 hours to TGF-β1 mediates a reversible growth arrest and does not alter the clonogenic capacity of keratinocytes (Shipley et al., 1986), and suggest that TGF-β1 does not induce apoptosis of stem and progenitor cells, even at high physiological concentrations.

The promotion of epidermal precursor amplification by low TGF-β1 concentrations (10-30 pg/ml) was particularly impressive in cultures initiated with primitive $Adh^{+++}EGF\text{-}R^{low}$ cells. Indeed, in the experiment shown in Fig. 2B, the mean output for one $Adh^{+++}EGF\text{-}R^{low}$ plated cell was 10^{11}-10^{12} in the control condition and this value reached 10^{16}-10^{17} in the cultures treated with 10 pg/ml TGF-β1, representing an increase from 38-39 to 55-56 PDs. Given that the initial cloning efficiency of $Adh^{+++}EGFR^{low}$ keratinocytes is less than ~2%

(results not shown), and making the assumption that only clonogenic cells contribute to the total cell output, we estimated that each clonogenic cell in this subpopulation can generate more than ~10^{18} keratinocytes (~60 PDs). One feature of the most primitive epidermal cells is their smaller size in comparison with more mature keratinocytes committed to differentiation (Barrandon and Green, 1985). As shown in Fig. 2C, a low concentration of TGF-β1 not only increased the cumulative keratinocyte expansion, but also prolonged the production of clones composed of small, undifferentiated keratinocytes. It is also important to note that TGF-β1-mediated expansion did not alter the organogenic potential of epidermal precursor cells, which showed a continuous capacity to form an epidermis equivalent to that of cells cultured without a low TGF-β1 concentration (results not shown).

Effects of low TGF-β1 concentrations on keratinocytes at the molecular level

TGF-β intracellular signaling is mediated through Smad, the mammalian homolog of the *Drosophila* Mothers against dpp (Mad). This cascade involves Smad1, Smad2 and Smad3 as direct targets of the type I TGF-β serine/threonine kinase receptor family (Abdollah et al., 1997; Liu et al., 1997; Souchelnytskyi et al., 1997; Chen et al., 1999) (reviewed by Moustakas et al., 2001). To determine whether concentrations of TGF-β1 as low as 10 pg/ml are able to activate the Smad pathway, we next analyzed the degree of Smad1 and Smad2/3 phosphorylation in cultures of keratinocytes, initiated with $Adh^{+++}EGF\text{-}R^{-/+}$ cells, and grown with or without 10 pg/ml TGF-β1. Phosphorylated forms of Smads, p-Smad1 (Ser463/Ser465) and p-Smad2 and 3 (Ser433/Ser435), were quantified and compared in each culture condition (Fig. 4). Results indicate that a treatment with 10 pg/m TGF-β1 is sufficient to increase Smad phosphorylation. In the representative control experiment shown, 47.2% and 26.1% of the keratinocytes were detected as positive cells for the presence of p-Smad1 and p-Smad2/3 respectively. These percentages reached respectively 76.3% and 54.3% in the presence of 10 pg/ml TGF-β1. Similarly, the median values of p-Smad1 and p-Smad2/3 fluorescence were respectively increased from 5.2×10^6 and 3.2×10^6 arbitrary units (a.u.), in the control condition, to 7.6×10^6 and 5.8×10^6 a.u. in TGF-β1-supplemented culture.

A high expression of α6 (CD49f) and β1 (CD29) integrin chains is known to be associated with an immature state of keratinocytes (Jones et al., 1995; Li et al., 1998). To investigate further the promotion of long-term amplification of epidermal precursors by low TGF-β1 concentrations, we analyzed and compared the expression of these cell-surface markers in long-term cultures initiated with $Adh^{+++}EGF\text{-}R^{-/+}$ cells and performed with or without 10 pg/ml TGF-β1. Typical labeling distributions obtained from cultures at passage 4 are presented in Fig. 5 (A-F). In the control culture, 53.1% and 66.1% of the keratinocytes were detected as positive cells for the expression of α6 and β1 integrins respectively (Fig. 5C,D). These percentages reached respectively 92.4% and 83.4% in the presence of 10 pg/ml TGF-β1 (Fig. 5E,F). Similarly, the median values of α6 and β1 integrin fluorescence were respectively increased from 9.5×10^6 and 11.3×10^6 a.u. in the control condition (Fig. 5C,D) to 16.7×10^6 and 16.5×10^6 a.u. in TGF-β1-supplemented culture (Fig. 5E,F), indicating a higher degree of immaturity in this optimized culture condition.

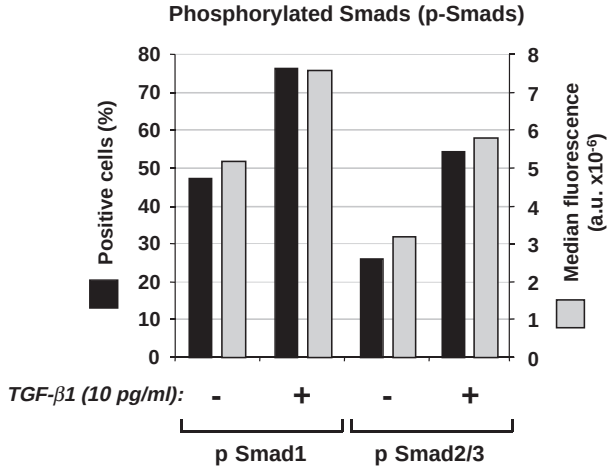

Fig. 4. Effect of a low TGF-β1 concentration on Smad1 and Smad2/3 phosphorylation. Keratinocytes from cultures initiated with $Adh^{+++}EGF\text{-}R^{-/+}$ cells were plated on glass slides and grown for 72 hours with or without 10 pg/ml TGF-β1. The degree of Smad1 and Smad2/3 phosphorylation (presence of p-Smad1 and p-Smad2 and 3) was then evaluated in each condition by immunofluorescence and compared by Laser Scanning Cytometry (LSC) analysis. Results from one representative experiment are presented (*n*=3). Data are expressed as percentages of positive cells and median values of fluorescence (arbitrary units, a.u.) detected in the two culture conditions.

Conclusions

We have developed a feeder layer-free culture system in which cell cycling of epidermal precursors can be transiently blocked by the addition of high TGF-β1 concentrations (⩾100 pg/ml), and keratinocyte expansion can be stimulated by the addition of very low TGF-β1 concentrations (10-30 pg/ml).

The involvement of TGF-β1 signaling in the control of the homeostasis of the epidermis has been confirmed in vivo in various transgenic mouse models (Cui et al., 1995; Wang et al., 1997; Amendt et al., 1998), and suggested in humans by clinical observations showing that a dysregulation of the TGF-β1 signaling cascade is often associated with the malignant conversion of skin keratinocytes (Lange et al., 1999). However, although these in vivo observations confirmed the inhibition of keratinocyte cell cycling described in vitro with high concentrations of TGF-β1, they do not provide any information about the positive effect of TGF-β1 on immature keratinocyte expansion, as demonstrated in this in vitro study with very low concentrations (10-30 pg/ml).

The potent biphasic effect of TGF-β1 previously reported for the development of hematopoietic progenitor cells (Fortunel et al., 2000a) and osteoclast-like cells (Shinar and Rodan, 1990) for in vitro angiogenesis (Pepper et al., 1993) and mammary gland ductal morphogenesis (Soriano et al., 1996) is here clearly demonstrated for human skin precursor cells. Functional studies may be applied in such culture systems to elucidate further the complex regulatory network involved in the exquisitely dose-dependent response of cells to

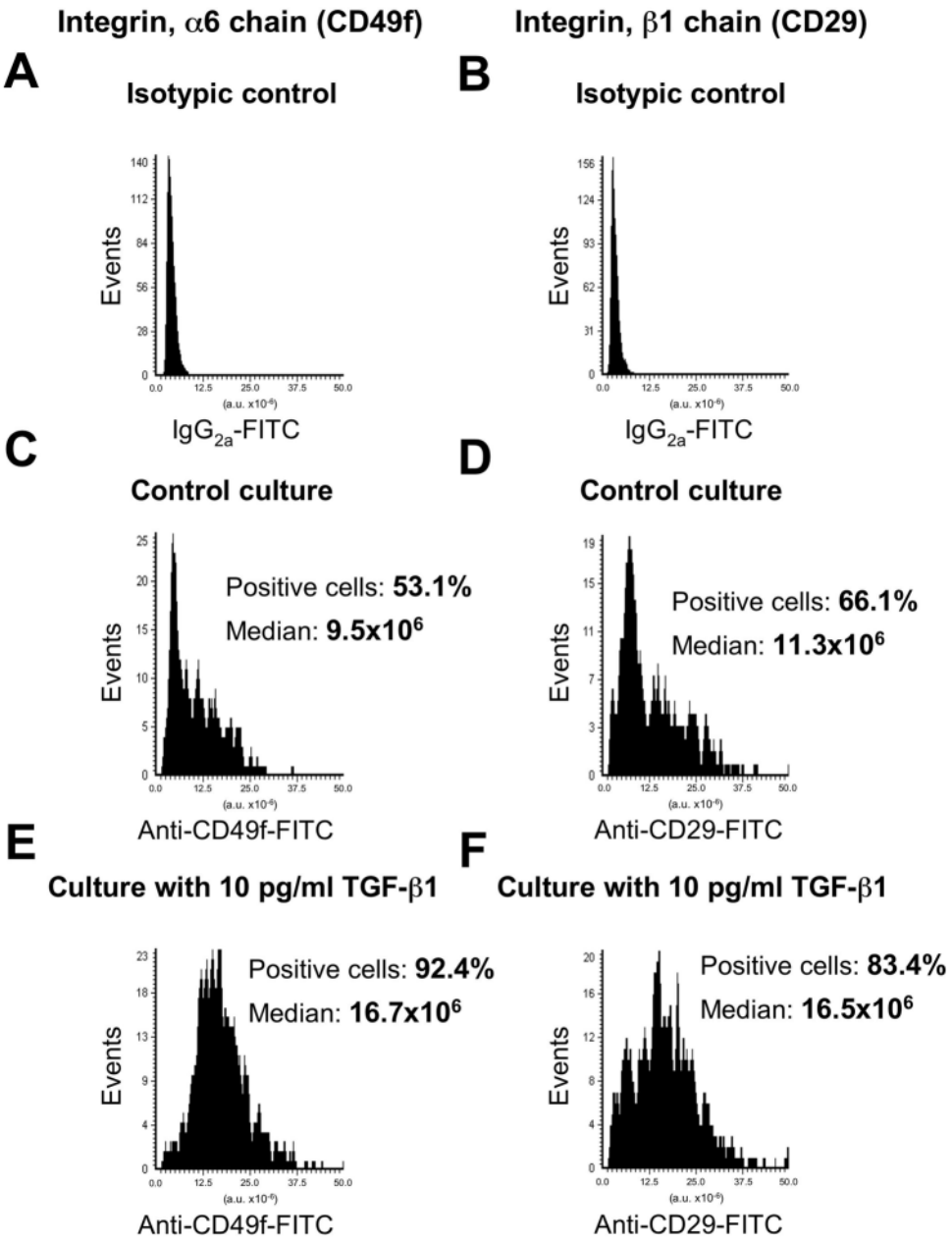

Fig. 5. Effect of a low TGF-β1 concentration on α6 (CD49f) and β1 (CD29) integrin expression by keratinocytes. Long-term cultures were initiated with $Adh^{+++}EGF\text{-}R^{-/+}$ cells and performed with or without 10 pg/ml TGF-β1. Cells at passage 4 were plated on glass slides, grown for 72 hours, and then processed for immunofluorescence. Expression of integrins was analyzed by Laser Scanning Cytometry (LSC) and compared in the two culture conditions. (A) Isotypic control corresponding to α6 integrin labeling. (B) Isotypic control corresponding to β1 integrin labeling. (C) α6 integrin expression profile of keratinocytes grown in the control condition. (D) β1 integrin expression profile of keratinocytes grown in the control condition. (E) α6 integrin expression profile of keratinocytes grown in the presence of 10 pg/ml exogenous TGF-β1. (F) β1 integrin expression profile of keratinocytes grown in the presence of 10 pg/ml exogenous TGF-β1. Representative labeling profiles from a typical experiment are presented (*n*=3).

TGF-β1. In addition to the various effectors regulating TGF-β1-induced cell-cycle arrest in keratinocytes (Hannon and Beach, 1994; Ivarone and Massagué, 1999), it will be interesting to focus on genes whose expression may be linked to the maintenance of the epidermal stem cell pool, such as $p21^{(WAF1/Cip1)}$ (Topley et al., 1999), 14-3-3σ (Dellambra et al., 2000), P63 (Pellegrini et al., 2000), c-Myc (Waikel et al., 2001), β-catenin (Zhu and Watt, 1999; Huelsken et al., 2001), genes involved in delta-notch signaling (Lowell et al., 2000), and the transcriptional regulators Tcf3 and Lef1 (Merril et al., 2001).

We describe here the capacity of low TGF-β1 concentrations to promote long-term expansion of undifferentiated human epidermal precursors, an effect that was suggested, but not demonstrated, in the hematopoietic system. However, much remains to be done to characterize this function at the molecular level. In an avian system, it has been reported that TGF-β1 is capable of sustaining erythrocytic progenitor cell proliferation and self-renewal, and that this effect occurs through a cooperation between the TGF-β and TGF-α receptors via the Mek-Map kinase pathway (Gandrillon et al., 1999). In the human hematopoietic system, TGF-β1 could participate in the maintenance of a pool of primitive and undifferentiated progenitors in part by upmodulating the immaturity marker, CD34, on cycling cells (Batard et al., 2000; Pierelli et al., 2000). TGF-β1 has not yet been demonstrated to regulate directly the expression of the mucin-like protein CD34 in normal hematopoietic stem/progenitor cells, but this has been demonstrated in the pluripotent erythroleukemia cell line TF-1. Marone et al., have shown that TGF-β1 transcriptionally activates CD34 and then prevents differentiation of TF-1 cells, by acting independently through Smad, TAK1 and p38

pathways (Marone et al., 2002). The downstream component of the Wnt signaling cascade, β-catenin, appears to be an important molecule especially in epidermal stem cells (Zhu and Watt, 1999; Huelsken et al., 2001). Thus, as described in the early amphibian embryo during the formation of Spemann's organizer (Nishita et al., 2000), a possible interaction between Wnt and TGF-β signaling should be investigated in the case of epidermal precursors treated with the low TGF-β1 concentrations described here to promote expansion.

One important clinical application of this work is the possibility of improving the culture systems currently used to amplify keratinocytes for skin grafting (Ronfard et al., 2000) by the addition of the TGF-β1 concentrations described here to optimize the long-term expansion of immature keratinocytes. Furthermore, the general principle applied here in purifying primitive cell subpopulations could be used as an approach to purify precursor cells from other somatic tissues. Similarly, since the manipulation of TGF-β1 appears to be effective in permitting in vitro expansion of primitive epidermal cells, it would be of interest to investigate whether similar use and manipulation of TGF-β1 might be applied in the expansion of other adult tissue precursors.

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