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Enrichment and identification of human 'fetal' epidermal stem cells

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BACKGROUND: Human epidermis, a continuously renewing tissue, is maintained throughout life by stem cells that proliferate and replenish worn-out or damaged cells in the tissue. Cultured human epidermal stem cells have great potential in clinical application. However, isolating and culturing a pure population of epidermal stem cells has proven to be challenging. METHODS AND RESULTS: We show that p63, a new marker for epidermal stem cells, is expressed in the basal layer of human fetal epidermis using immunohistochemistry, and that keratinocytes with the characteristics of stem cells can be isolated from the epidermis of aborted human fetuses aged ⩾20 weeks based on high expression of β_1 integrins by fluorescence-activated cell sorting. Furthermore, the enriched population showed the expression of molecular markers of putative human epidermal stem cells under a confocal microscope and a high colony formation efficiency when it was cultured at a clonal density. Under an electron microscope the sorted stem cells exhibited a high nuclear:cytoplasmic ratio and fewer organelles than the transit amplifying cells. The cultured epidermal stem cells can also be amplified and induced to terminal differentiation by suspension *in vitro*. CONCLUSIONS: Human 'fetal' epidermal stem cells have been successfully isolated and cultured *in vitro*. The cultured human epidermal stem cells could be used as a tool for studying stem cell biology and testing stem cell therapy.

Key words: CFE/epidermal stem cell/FACS/integrin β_1/p63

Introduction

Human epidermis, a stratified squamous epithelium, is made up of multiple layers of keratinocytes. A subpopulation in the basal layer, known as epidermal stem cells, give rise to transit amplifying (TA) cells by symmetric or asymmetric division (Watt and Hogan, 2000). The interfollicular epidermis, hair follicle and sebaceous gland all arise from TA cells (Taylor *et al*., 2000). Epidermal stem cells play a central role in homeostasis and wound repair and also represent a major target of tumour initiation and gene therapy (Fuchs and Raghavan, 2002; Owens and Watt, 2003; Perez-Losada and Balmain, 2003). It has been shown recently that mouse epidermal stem cells could produce multiple cell lineages during development and it was proposed that the skin could be a source of easily accessible stem cells that are able to be reprogrammed to form multiple cell lineages (Liang and Bickenbach, 2002).

Isolation and identification of putative human epidermal stem cells has been done previously. In earlier studies, it has been found that *in vitro* three types of colony, holoclone, paraclone and meroclone, could be formed because of the different amplification potentials of human keratinocytes (Barrandon and Green, 1987). Holoclones are thought to be derived from epidermal stem cells, while the other two types of clones are from TA cells. Although this assay is very useful to investigate the proliferative potential *in vitro*, the comparison between these cultures is hard to make. Keratinocytes with characteristics of stem cells can be isolated on the basis of high surface expression of β_1 integrins and rapid adhesion to extracellular matrix proteins (Jones and Watt, 1993). Stem cells can be isolated to >90% purity by fluorescence-activated cell sorting (FACS) using β_1 integrin as a molecular marker. However, human epidermal stem cells expressed ~2-fold higher levels of β_1 integrin than TA cells, which make it difficult to reliably purify stem cells using FACS. Recently, p63 transcription factor, a p53 homologue, has been shown to be a secific marker for keratinocyte stem cells. It helps distinguish human keratinocyte stem cells from their TA progeny (Mills *et al*., 1999; Pellegrini *et al*., 2001). β-Catenin is a major structural component of adherens junctions and a downstream effector of the Wingless (*Drosophila*)/Wnt (vertebrate) signalling pathway (Wiechaus and Riggleman, 1987; Ben-Ze'ev and Geiger, 1998). The phosphorylated β-catenin, reflecting Wnt signalling activities, appears to be expressed at higher levels in cultured epidermal stem cells than TA cells (Zhu and Watt, 1999).

Previous studies have shown that in the process of serial cultivation of human keratinocytes, the plating efficiency and culture lifetime were inversely related to the age of the skin

donor (Rheinwald and Howard, 1975). However, integrin β_1 expression does not change in abundance or distribution during skin development (Hertle *et al*., 1991). Therefore, we have hypothesized that the fetal epidermis may contain more stem cells than the adult epidermis. To validate our hypothesis, we have isolated and characterized epidermal stem cells from human fetuses.

In this study, we have examined the expression of p63 in the human fetal skin using immunofluorescent histochemistry. The epidermal stem cells have been isolated from human fetal epidermis of different ages by FACS based on expression of integrin β_1. The purified epidermal stem cells have been cultured *in vitro* and further characterized for expression of molecular markers, colony formation efficiency (CFE) and differentiation potential.

Materials and methods

Tissue preparation and immunohistochemistry

Aborted human fetuses (n = 15) were collected from a local hospital and used under the permission of the ethical committee of the Institute of Zoology, Chinese Academy of Sciences (CAS). The human fetal epidermis were harvested and stored at –80°C. Tissue sections were made at 8 μm on a cryostat and stored at –80°C. For immunohistochemistry, slides were fixed with 4% paraformaldehyde for 1 h at room temperature, rinsed with phosphate-buffered saline (PBS) for three times, treated with Triton-100 for 10 min, rinsed with PBS twice, and incubated with a primary antibody overnight at room temperature. After washing with PBS three times, the appropriate fluorescein isothiocyanate (FITC)-labelled secondary antibody was added and incubated for 45 min at 37°C. After many washes in PBS, propidium iodide (PI, 1 μg/ml) was added and incubated for 2 min at room temperature. The slides were washed again with PBS and examined under a confocal microscope.

Isolation and cultivation of human fetal keratinocytes

The basal keratinocytes were isolated after digestion with 0.25% trypsin and 0.02% EDTA for overnight at 4°C, then cultured by using a mitomycin C-treated NIH3T3 cell as the feeder using the method as previously described (Jones and Watt, 1993). The medium was made up of three parts of Dulbecco's modified Eagle's medium plus one part of Ham's F-12 medium containing 10% fetal calf serum (Hyclone), 20 ng/ml epidermal growth factor (Sigma), 5 μg/ml transferrin (Sigma), 1.8×10^{-4} mol/l adenine (Amresco), 5 μg/ml insulin (Sigma), 10^{-10} mol/l cholera toxin (Sigma) and 0.4 μg/ml hydrocortisone (Sigma). The cultures were grown in a humidified 5% CO_2 atmosphere at 37°C and the medium changed every 2 days. Keratinocytes were passaged at 80% confluence after removing the feeder cells with 0.02% EDTA.

Antibodies

The final concentration of FITC-labelled anti-integrin β_1 mouse monoclonal antibody (sc-9970 FITC; Santa Cruz) and isotype-matched negative control antibody (sc-2855; Santa Cruz) were used at 1 μg/ml in the FACS. The anti-p63 antibody (sc-8431; Santa Cruz), anti-involucrin antibody (sc-15223; Santa Cruz), anti-nestin antibody (MAB353; Chemicon) and anti-active-β-catenin antibody (05-665; Upstate) were used in the immunofluorescent staining at dilutions suggested by the suppliers.

Cell preparation for FACS

Newly confluent cultures at first passage were used for staining. Cells were harvested by removing the feeders first with 0.02% EDTA and then treating the keratinocyte with trypsin–EDTA. After washing with 1% bovine serum albumin (BSA), the cells were then incubated in 1% BSA with the FITC-labelled anti-integrin β_1 antibody for 1 h at 37°C. After washing with 1% BSA three times, cells were filtered with a cell mesh (70 μm). Immediately before analysis, PI (1 μg/ml) was added for viability gating. Cells were kept on ice until the flow cytometric procedure.

Flow cytometry

Cells were analysed on a Becton–Dickinson FACScan and sorted using a Cytomation Plus (Dako). For cell sorting, gates were set using forward light scatter (FSC) and side light scatter (SSC). A total ⩾f 50 000 events was acquired in list mode for each sample. The integrin fluorescence was detected on the FL1 channel (520–560 nm band pass filter). PI was excited with 100 mW of UV radiation (351–364 nm). Debris and cells positive for PI were gated out.

Electron microscopy

To observe the morphology of stem cells, the sorted cells were fixed with glutararaldehyde. Then the samples were prepared and treated according to standard procedures. Samples were observed under a TEM (H-7500; Hitachi) and results recorded.

Cultivation of sorted epidermal stem cells

Sorted epidermal stem cells have been cultured on a collagen type IV-coated (100 μg/ml) plate. After sorting, the epidermal stem cells were seeded into a 24-well plate at a density of 10^5 cells per well. The medium was as for those mentioned above and was changed every 2 days.

Determination of CFE

To determine the CFE of sorted populations, the sorted cells were seeded in 6-well plate at a density of 1000 cells/well. The plate was seeded with mitomycin C-treated feeders 24 h before use and the medium changed every 2 days. After culturing for 2 weeks, these cells were fixed with 4% formaldehyde, washed in PBS, and stained with 1% Rhodamine B (Sigma) (James and Howard, 1975). Colonies were then counted under a microscope. Only colonies >32 cells were scored. The CFE was designated as the number of colonies divided by the total number of seeded cells.

Immunocytochemistry

For the immunocytochemistry analysis of sorted cells, cells were air-dried onto glass slides, fixed with a mixture of methanol and acetone (1:1) for 10 min at room temperature, rinsed in PBS for three times, and incubated with an primary antibody at 4°C for overnight. The other steps were the same as described above for immunohistochemistry.

Suspension-induced terminal differentiation

Suspension-induced terminal differentiation was performed as previously described (Green, 1975). In brief, the sorted cells were placed in a medium containing 1.6% methyl cellulose (Sigma). After 24 h at 37°C, the cells were harvested and used for immunocytochemistry analysis.

Statistics

Results were presented as the average ± SD of at least three independent experiments. Statistical differences were evaluated by analysis with t-test; values of $P < 0.05$ were accepted as significant.

Results

Expression of p63 in the human fetal epidermis

In all fetal epidermis studied from fetuses of 20–34 weeks gestation, the expression of p63 was mainly confined to the basal layer, a place where stem cells have been believed to be located. Interestingly, p63 was also present in the developing sweat glands (Figure 1).

Cultivation of the human fetal basal keratinocytes

fetal basal keratinocytes were isolated from many aborted human fetuses (n = 15). In the process of isolating basal keratinocytes, we observed that fetal epidermis could be easily separated from the dermis of fetuses which were aged >20 weeks only. In the younger fetuses, more fibroblast cells easily contaminated the culture because of the poor separation between epidermis and dermis. The 'contaminating' fibroblast cells could take over the cultures and prevent the growth of keratinocytes a few days after culturing. The isolated human fetal basal keratinocytes grew very well on the mitomycin C-treated 3T3 feeder. Furthermore, in the cultures, three types of colony, holoclone, paraclone and meroclone, were observed. Among the colonies, most of them were holoclones with high amplification potential. Some stratified cells were observed in the center of the colony (Figure 2a). After culturing for >2 weeks, the keratinocytes from different fetuses showed no obvious difference in the cell morphology and multiplication potential. The culture could be passaged several times and maintained a high level of integrin β_1 expression (see Figure 2b and c).

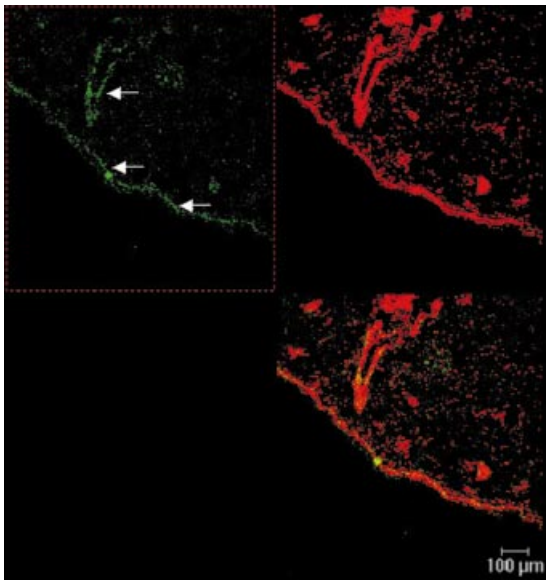

Figure 1. Expression of p63 protein in the basal layer of human fetal epidermis. The green colour represents p63 protein staining (indicated by the arrow) and the red colour indicates nucleus staining. The yellow colour represents the overlap of green and red. Scale bar = 100 μm.

Epidermal stem cells isolation by flow cytometry

As shown in Figure 3a, cells with a lower FSC and SSC have been gated in region R1. Dead cells were gated out using PI. The cells in region R1 were mostly basal keratinocytes and the rest were suprabasal cells. The population in region R1 showed a single peak of fluorescence after it was stained with a FITC-labelled anti-integrin β_1 antibody (Figure 3b). Those cells in region R2 with the highest integrin β_1 level, ~10% of basal keratinocyte, were sorted as putative epidermal stem cells. At the same time, a population with a lower integrin β_1 expression level in region R3 were sorted as TA cells (Figure 3b). The sorted cells were used for cultivation, CFE determination and molecular marker expression analysis.

Molecular marker expression in the fetal epidermal stem cells

To evaluate the various sorted cell populations, we examined the expression of some previously reported markers in the human epidermal stem cells using immunofluorescence. Under a fluorescent microscope, integrin β_1 (Figure 4a) and active β-catenin (Figure 4b) were highly expressed on all of the sorted cells. The newly reported marker p63 was also expressed in the nucleus of sorted putative stem cells (Figure 4c). These results showed that this sorted population likely represented the epidermal stem cell population. In addition, we have found that nestin, a putative marker, appeared widely in neural stem cells

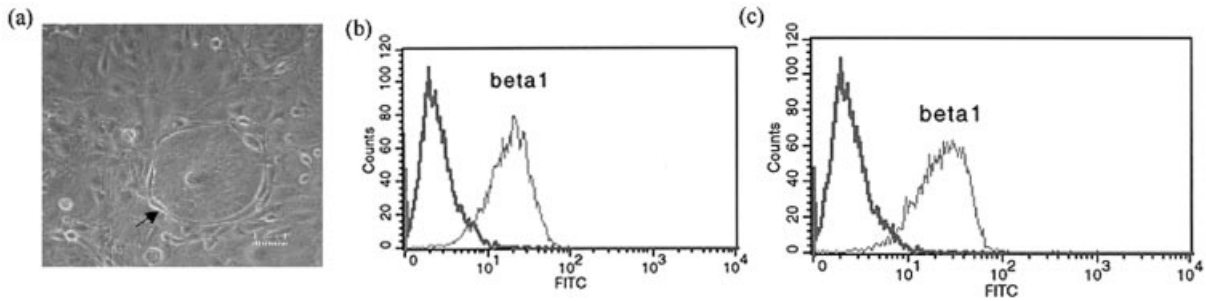

Figure 2. Culturing and expression of integrin β_1 in the human fetal basal keratinocytes. (**a**) Colony produced by human fetal basal keratinocytes (indicated by the arrow). Cells around the colony are mitomycin C-treated NIH 3T3 cells used as feeder cells. (**b**) Integrin β_1 expression in human fetal basal keratinocytes of passage 2. (**c**) Integrin β_1 expression in human fetal basal keratinocytes of passage 7. The right peak shows the staining with FITC-labelled anti-integrin β_1 mouse monoclonal antibody. The left peak shows the staining with isotype-matched negative control antibody.

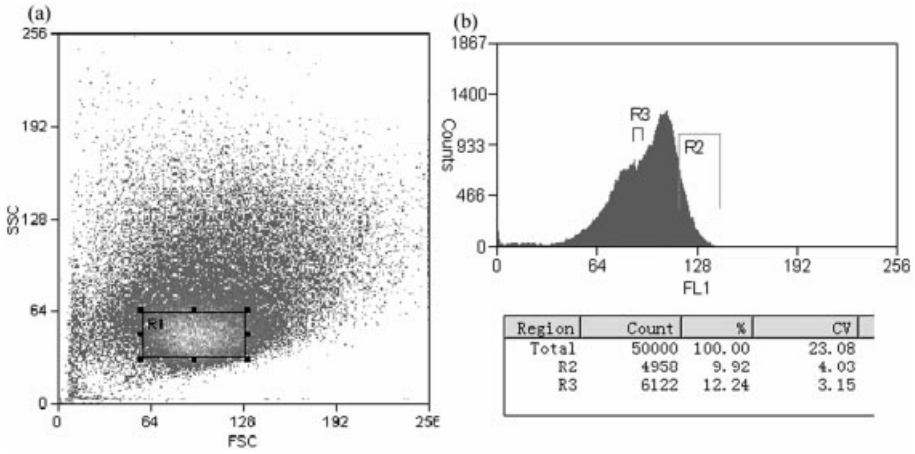

Figure 3. Fluorescence-activated cell sorting of putative human fetal epidermal stem cells. (**a**) Dot plot shows the FSC (forward light scatter) and SSC (side light scatter) of a suspension of cultured fetal basal keratinocytes. Cells with lower FSC and SSC are gated in region R1. (**b**) Basal keratinocytes selected on the basis of FSC and SSC in region R1 are stained with a FITC-conjugated antibody to integrin β_1 and examined in a flow cytometer. Cells in region R2 with the highest integrin β_1 level are sorted as epidermal stem cells. A population with lower integrin β_1 in region R3 are sorted as transit amplifying (TA) cells.

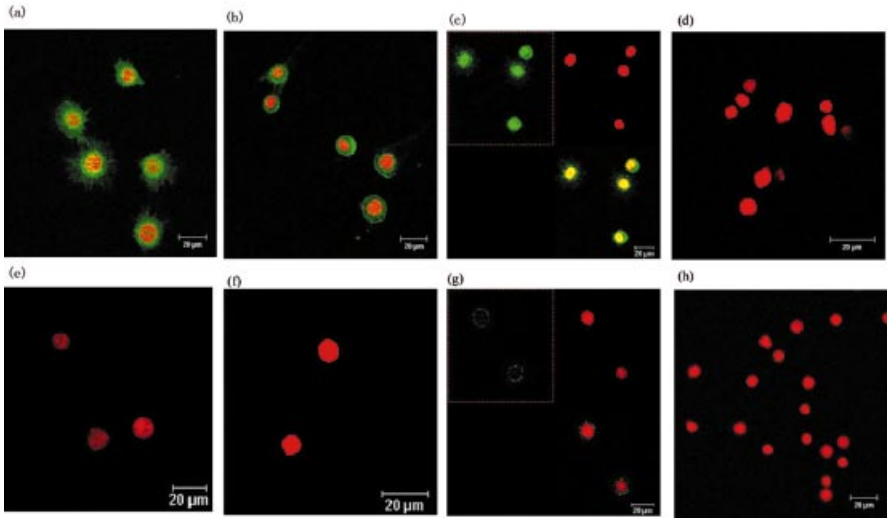

Figure 4. Expression of molecular markers in the sorted epidermal stem cells. (**a**) Expression of integrin β_1 in the sorted epidermal stem cells. (**b**) Expression of β-catenin in the sorted epidermal stem cells. (**c**) Expression of p63 in the sorted epidermal stem cells. It presents in the nucleus of the cells. (**d**) Non-expression of nestin in the sorted epidermal stem cells. (**e**–**h**) Negative control of **a**–**d** respectively. Scale bar = 20 μm.

and other adult stem cells, but was not expressed in the sorted epidermal stem cells (Figure 4d).

Morphology of sorted epidermal stem cells

It was believed that genuine stem cells showed a large nuclear:cytoplasmic ratio. In our study, we have also found that the sorted stem cell population and TA cells exhibited differences in cytological characteristics and cell size. The putative stem cell population contained significantly smaller cells than the TA population. Moreover, under an electron microscope the putative stem cells exhibited a high nuclear: cytoplasmic ration consistent with undifferentiated or primitive cells (Figure 5). Most of the putative stem cells have a large nucleus and small cytoplasm with few cytoplasmic organelles (Figure 5a). The TA cells have a smaller nucleus but large cytoplasmic portion with many cytoplasmic organelles (Figure 5b). These data indicated that the sorted putative stem cell population have characteristics of immature cells.

Proliferation of sorted epidermal stem cells

To evaluate whether the sorted stem cell population could be cultured and proliferated *in vitro*, we have tried to culture it in a system without the 3T3 feeder cells. In our study, we found that the stem cells required a higher than normal concentration of cells to grow in culture after the physical stress of sorting (~10^5 cells per well of a 24-well plate). After seeding in a collagen type IV-coated plate, ~95% of the stem cells adhered to the bottom within 20 min. The stem cell grew very slowly at first and after 6 days it began to grow quickly, taking ~10 days to reach confluence (Figure 6). But the TA cells grew very rapidly

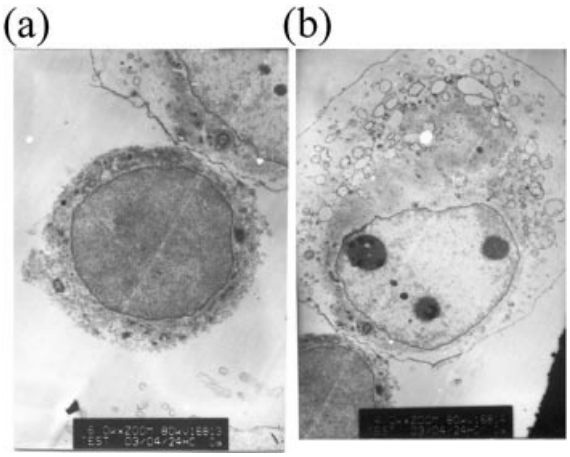

Figure 5. Electron microscope image of stem and transit amplifying (TA) cells. (**a**) Stem cells, which possess a large nuclear portion and small portion of cytoplasm with few organelles. (**b**) TA cells, which possess a smaller nucleus but a large cytoplasm with many organelles.

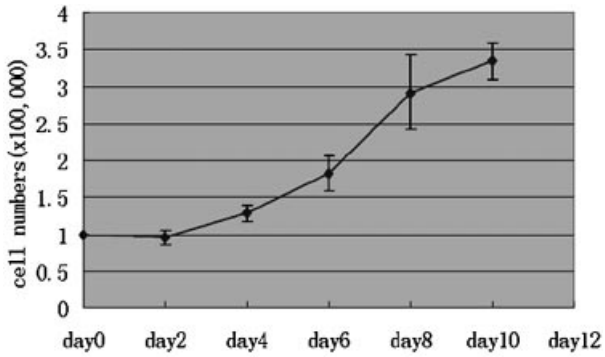

Figure 6. Cell growth of sorted epidermal stem cells. At first, sorted stem cells were planted into a collagen type IV-coated plate at a concentration of 10^5 cells per well of 24-well plate. After culturing for the required number of days, cells were digested and counted under a phase microscope. Data represent the mean ± SD from three independent experiments.

and reached confluence in <7 days (data not shown). These data suggest that stem cells may be slower to enter a proliferative phase than TA cells.

CFE of the sorted fetal epidermal stem cells

Immediately after sorting, the cells were plated onto feeder cells to determine the CFE. After 14 days of culture, the colonies could be scored under the microscope after Rhodamine B staining. As shown, two enriched populations were also able to form colonies (Figure 7a–c). The centres of the colonies were strongly stained because of the stratification (Figure 7c), so that each colony could be easily counted under a phase microscope. When 1000 cells were seeded onto a 35 mm dish containing mitomycin C-treated 3T3 feeder cells, the stem cells could form many more colonies than TA cells). The putative epidermal stem cells had a CFE of 35.6 ± 5.4%, but the TA cells had a much lower CFE, 4.5 ± 1.1% (Figure 7d, $P < 0.05$). This observation suggested that sorted putative stem cells have a higher proliferative potential than TA cells.

Suspension-induced terminal differentiation of sorted epidermal stem cells

To determine whether the sorted fetal epidermal stem cells can undergo terminal differentiation, we placed the sorted population on a medium containing 1.6% methyl cellulose to be cultured in suspension. After culturing for 24 h, compared with control cells which had not been induced (Figure 8a), almost all the cells expressed involucrin (Figure 8b), which is the marker indicating the keratinocyte terminal differentiation (Adams and Watt, 1989). These results indicate that the sorted fetal epidermal stem cells can undergo terminal differentiation as normal stem cells do.

Discussion

Our study shows for the first time that p63 is expressed in the basal layer of human fetal epidermis, where epidermal stem cells are supposed to be located (Jones *et al*., 1995). Then the putative 'fetal' epidermal stem cells were isolated from the epidermis of abortive human fetuses (n = 15) using FACS based on high surface expression of integrin β_1.

There are at least two factors important for isolating epidermal stem cells. One is choosing specific surface molecular markers, and the other is separating basal keratinocyte population containing epidermal stem cells from the rest of the epidermis. In addition to integrin β_1, integrin α_6 has also been suggested to be a better stem cell marker (Li *et al*., 1998). In fact, lack of specific molecular markers has been a major obstacle for stem cell research (Lavker and Sun, 2000; Tani *et al*., 2000). In the mouse, side population (SP) cells have been isolated as genuine epidermal stem cells using FACS and Hoechst staining (Dunwald *et al*., 2001; Liang and Bickenbach, 2002). But in humans, our study suggested there were no SP cells in the epidermis (Zhou JX, Jia LW, Liu WM, Liu S, Cao YJ, Duan EK unpublished data). This study has shown that integrin β_1 can function as a reliable marker for isolating human epidermal stem cells.

We show that the sorted putative epidermal stem cells express integrin β_1, p63 and phosphorylated β-catenin, but not nestin. Identification of human epidermal stem cells mainly depended on two parameters, molecular marker expression pattern and CFE determination (Lavker and Sun, 2000; Watt, 2001). In addition to integrinβ_1, p63 has served as an important marker for epidermal stem cells (Mills *et al*., 1999; Pellegrini *et al*., 2001). Active β-catenin has been shown to contribute to the high proliferative potential of keratinocytes with the characteristics of epidermal stem cells (Zhu and Watt, 1999). Our sorted putative epidermal stem cells have been further verified by p63 and active β-catenin expression and high CFE potential. We also tried to investigate whether nestin, a widely distributed marker of adult stem cells (Lendahl *et al*., 1990; Hunziker and Stein, 2000), appeared in the sorted epidermal stem cells. The results suggested that it did not appear in our sorted epidermal stem cells. The sorted epidermal stem cells could proliferate *in vitro* although perpaps they are slow to enter a proliferative phase. However, it appears that the CFE of

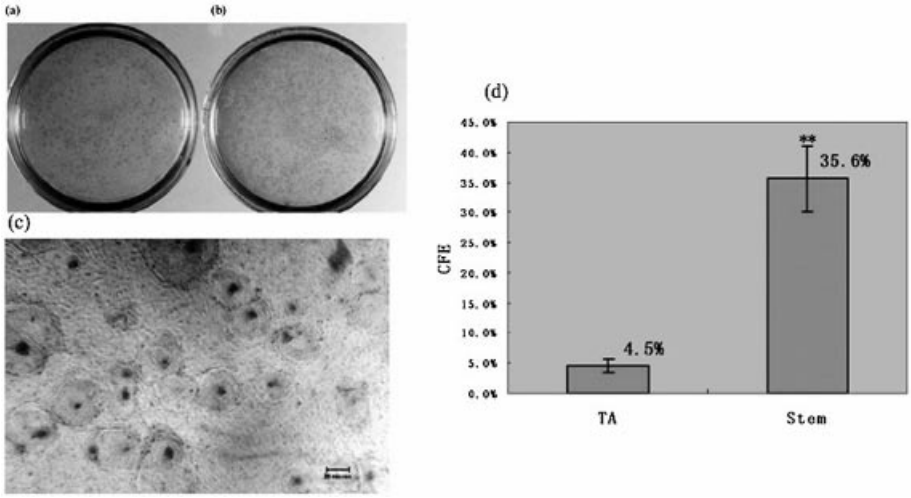

Figure 7. Morphology of the colonies and colony formation efficiency (CFE) of sorted stem and transit amplifying (TA) cells. Morphology of the colonies from TA cells (**a**) and stem cells (**b**) after staining with Rhodamine B. (**c**) Partial amplified morphology of the colonies from stem cells. Centre of the colony was strongly stained because of the stratification. (**d**) Comparison of CFE between the stem cells and TA cells. CFE of sorted stem cells (35.6 ± 5.4%) is significantly higher than that of the TA cells (4.5 ± 1.1%) (**$P < 0.05$).

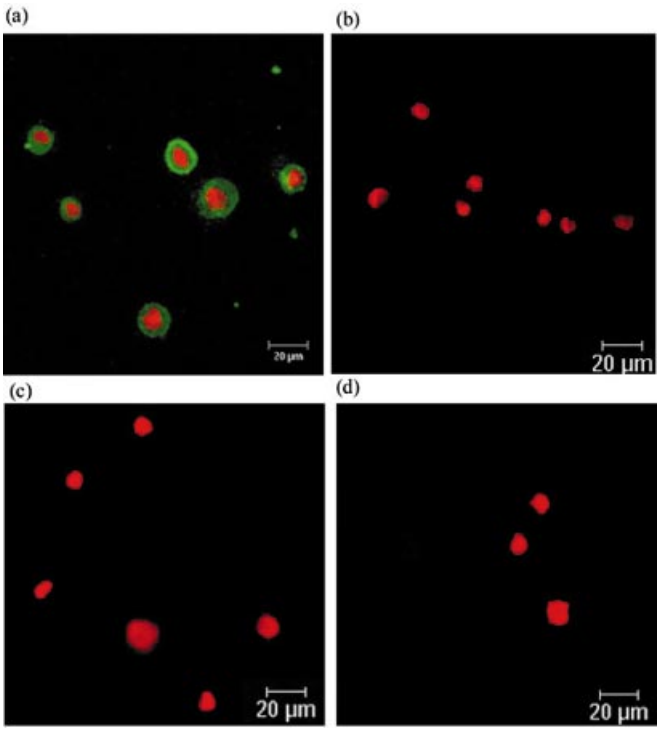

Figure 8. Expression of involucrin in suspension-induced epidermal stem cells. (**a**) Stem cells after a suspension inducement. After a culture in suspension, the stem cells expressed involucrin. (**b**) Stem cells free from a suspension inducement did not express involucrin. (**c**–**d**) Negative controls of **a** and **b** respectively. The green colour represents involucrin protein staining and the red colour indicates nucleus staining. Scale bar = 20 μm.

our sorted putative stem cells was lower than that previously described (Jones and Watt, 1993; Janes *et al*., 2002). This may imply that our sorted stem cells were less pure. It may also be due to sorting efficiency and/or difference in integrin β_1 expression between fetal and adult epidermal cells. Finally, the cultured stem cells can be induced to generate differentiated cells, which show the expression of involucrin.

In conclusion, epidermal stem cells have been successfully isolated from human fetal epidermis using FACS and expanded *in vitro*. The isolated epidermal stem cell population will be extremely useful in the future for studying stem cell biology and the potential of generating functional differentiated cells other than epidermal cells.

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